Clinical Application of Adriamycin Resistance Screening And The *In Vitro* Effect of Adriamycin on Osteosarcoma Cells

by

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ABSTRACT

Osteosarcoma is the most common primary malignant tumour of bone apart from myeloma. Many investigators are searching for prognostic factors to monitor the response of tumours to chemotherapy. Current studies focus on evaluating the reliability of P-glycoprotein expression using immunohistochemistry data. However, its association with response to chemotherapy is controversial since P-glycoprotein immunohistochemistry may only help to detect multidrug resistance in highly resistant tumours. Baldini and his co-workers developed the adriamycin binding assay which is a clinical assay and enables detection of low levels of resistance in tumour cell cultures. In this study, the applicability of P-glycoprotein immunohistochemistry and that of the adriamycin binding assay to predict tumour necrosis were compared.

We found that 46.15% of osteosarcoma samples studied had intrinsic adriamycin resistance as revealed by the adriamycin binding assay. However, P-glycoprotein expression was low in these samples. P-glycoprotein expression was elevated after chemotherapy, which may be the result of increased rate of synthesis. Pre-chemotherapy adriamycin binding assay results correlate at a statistically significant value ($p=0.042$) with tumour necrosis. Post-chemotherapy adriamycin binding assay results and P-glycoprotein expression did not have a statistically significant correlation with tumour necrosis. Moreover, the correlation between adriamycin sensitivity and P-glycoprotein expression in our study was not statistically significant.

In our osteosarcoma samples, P-glycoprotein expression is not the main reason for drug resistance as suggested by the adriamycin binding assay. Alternative mechanisms for drug resistance have been demonstrated. Adriamycin is known to impose growth inhibition on cancer cells. However, its effect on the activities of antioxidative enzymes is controversial. We postulate that intracellular antioxidants might play a role in modulating drug response in osteosarcoma cells. Therefore, we extended our study using an *in vitro* model to investigate the effect of adriamycin on the proliferation rate and the activities of two critical antioxidative enzymes, catalase and glutathione

peroxidase, in an osteosarcoma cell line. In addition, the adriamycin binding assay and P-glycoprotein immunohistochemistry were used to monitor the change of drug sensitivity during drug treatment in this model.

Our *in vitro* study showed that after adriamycin treatment up to 40ng/ml, the osteosarcoma cells developed adriamycin resistance, yet P-glycoprotein expression was not induced. The proliferation rate and the activities of antioxidative enzymes were all suppressed after incubating the cells with sub-concentration of adriamycin. The decrease in proliferation rate and antioxidative enzymes activities was postulated to be due to an inhibition by excess superoxide ions generated by adriamycin through quinone reduction.

In this study, we have clearly demonstrated that pre-chemotherapy adriamycin binding assay is critical for screening out patients who are intrinsically drug resistant. We also find that P-glycoprotein overexpression and enhanced activities of antioxidative enzymes may not be the rasons for adriamycin resistance. Further studies on other mechanisms should be conducted.

撮文

骨肉瘤是除骨髓瘤之外最普遍的原發性惡性骨腫瘤。很多研究員正尋找預後因子用以監察腫瘤在化療期間對藥物的反應。當前研究集中在評估 P-醣蛋白免疫組織化學數據的可信性，但其與藥物反應的關係仍具爭議。而 P-醣蛋白免疫組織化學只能偵測高抗藥性腫瘤對多種藥物的抵抗。Baldini 及其同僚發展了紅霉素結合測試。這項測試已被証明能有效地偵測出培養中癌細胞的低抗藥性。這次研究中，我們比較了 P-醣蛋白免疫組織化學及紅霉素結合測試在預測腫瘤壞死方面的應用能力。

這次研究中紅霉素結合測試顯示百分之 46.15 骨癌樣本原本已具有抗紅霉素能力。這些樣本均有低 P-醣蛋白的表達。P-醣蛋白的表達在化療後可能因其合成加快而上升。化療前紅霉素結合測試結果與腫瘤壞死在統計學上有顯著相關。化療後紅霉素結合測試結果及 P-醣蛋白的表達與腫瘤壞死在統計學上沒有顯著相關 。此外，在我們的骨肉瘤樣本中紅霉素敏感度與 P-醣蛋白的表達在統計學上亦沒有顯著相關。

在我們的骨肉瘤樣本中，P-醣蛋白的表達並非抗藥性的主要原因。先前已有報告指出抗藥性可能由於很多機制，亦已知紅霉素能抑制癌細胞生長，但它對抗氧化酶活性的影響則仍具爭議。我們推測細胞內的抗氧化劑可能幫助調整骨肉瘤細胞的藥物反應，因此我們用一個試管模型來研究紅霉素對骨肉瘤細胞分裂速度及兩種抗氧化酶：催化酶和谷胱甘肽過氧化酶的影響。而紅霉素結合測試及 P-醣蛋白免疫組織化學則用來監察細胞對藥物敏感度的改變。

我們的模型研究顯示經多至 40ng/ml 紅霉素處理後，腫瘤細胞變得抗紅霉素，但 P-醣蛋白的表達並未被誘發。細胞經低濃度紅霉素處理後分裂速度及抗氧化酶的活性均被制止。細胞分裂速度及抗氧化酶活性的下降可能是由於紅霉素醌還原產生過多超氧化離子所致。

是項研究中，我們清楚証明了化療前的紅霉素結合測試能過濾原本已對藥物有抵抗力的病人。我們亦發現過量 P-醣蛋白的表達及抗氧化酶活性的提高可能並非抗紅霉素的原因。未來的研究應考慮其他機制。

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ABBREVIATIONS

ABA	Adriamycin Binding Assay
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
GPx	Glutathione Peroxidase
GSH	Glutathione, reduced
GSSG	Glutathione, oxidized
HNE	4-Hydroxynonenal
IMDM	Iscove's Modified Dulbecco's Medium
MDR	Multidrug Resistance
P-gp	P-glycoprotein
PBS	Phosphate Buffered Saline
PSN	Penicillin-Streptomycin-Neomycin
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase

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1. INTRODUCTION

1.1. Osteosarcoma

According to the World Health Organization, osteosarcoma is defined as "a malignant tumour characterized by the direct formation of bone or osteoid by the tumour cells" (Schajowicz, 1993). It is the most common primary malignant tumour of bone apart from myeloma (Figure 1.1.). Osteosarcoma can be divided into two fundamental groups, namely the central (medullary) and the surface (peripheral) osteosarcomas. Intracortical osteosarcomas are extremely rare (Table 1.1.).

1.1.1. Incidence

The annual incidence of osteosarcoma in Chinese population is 0.23 cases per 100,000 people (Bovill *et al.*, 1975). In Hong Kong, its incidence is approximately 0.1 per 100,000. In 1992, 41 males and 20 females were newly diagnosed with osteosarcoma. 16 male and 10 female patients died of the disease (Hospital Authority, 1996). In the Prince of Wales Hospital about 10 new cases of osteosarcoma are diagnosed each year.

The specific cause of osteosarcoma is not known. Evidence suggests that osteosarcoma can run in families. Associations with other diseases (many of which are hereditary) and recent advances in recombinant DNA technology have identified genes that indicate an increased risk. The gene involved in familial retinoblastoma appears to be a defective tumor inhibitor gene and is associated with other tumors including osteosarcoma.

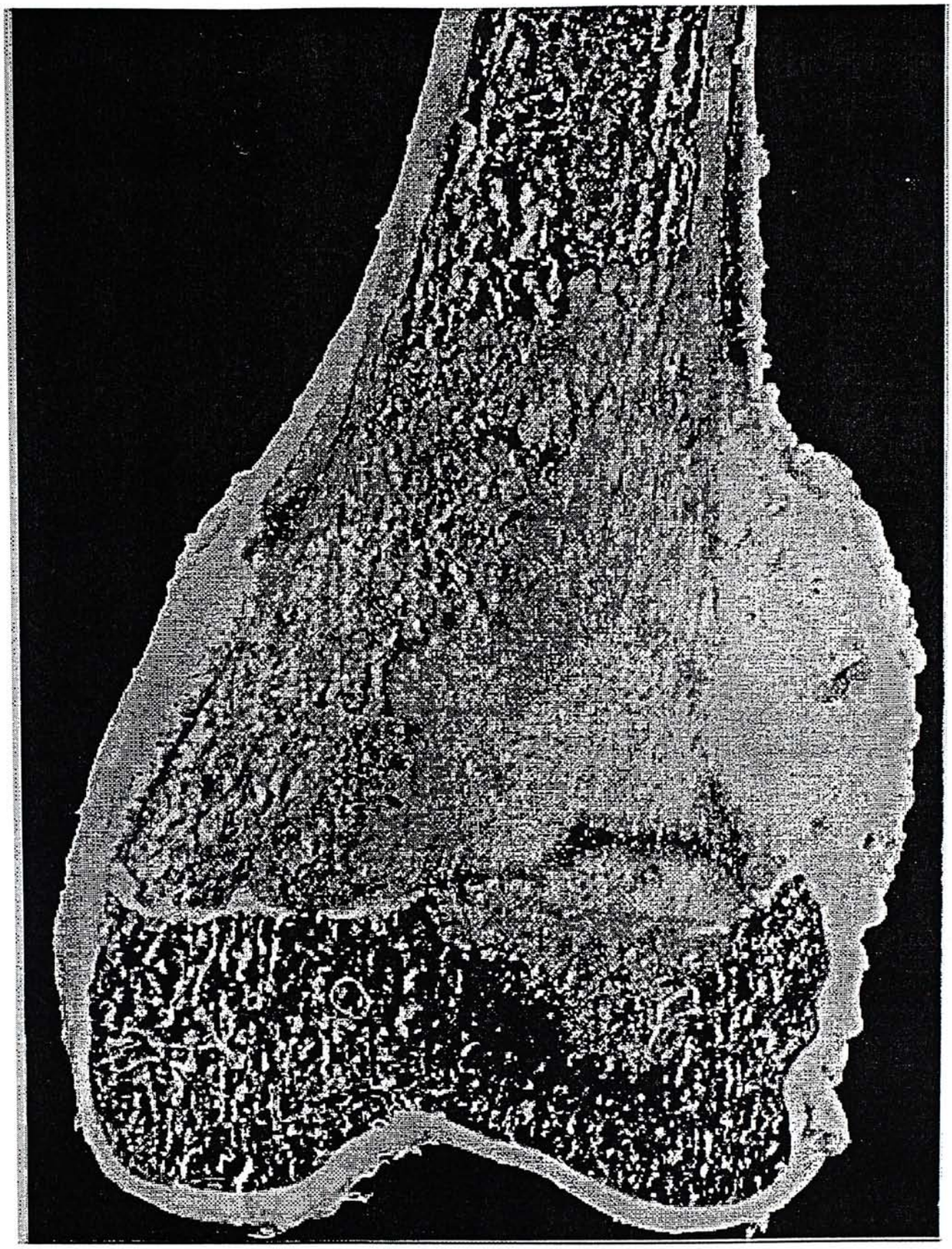


Figure 1.1. Osteosarcoma of the lower metaphysis of femur in a 13 years old male. The tumour extended into the epiphysis with periosteal new bone formation (Schajowicz, 1994).

Central (Medullary) Osteosarcoma	*Surface (Peripheral) Osteosarcoma*
Conventional Central Osteosarcoma	Parosteal (Juxtacortical) Osteosarcoma
Telangiectatic Osteosarcoma	Periosteal Osteosarcoma
Intraosseous Well-Differentiated Osteosarcoma	High-Grade Surface Osteosarcoma
Round-Cell Osteosarcoma	

Table 1.1. Classification of osteosarcoma (Schajowicz, 1993). Osteosarcoma occurs most frequently in on bone surface or in the medullary. Intramedullary osteosarcomas are rare.

1.1.2. Age and Sex Distribution

Osteosarcoma can occur at any age, although it mainly affects children. Most patients present with osteosarcoma during the first two decades of life, and patients between 10 and 20 are by far the most commonly affected (Wilimas *et al.*, 1977). The mean age of patients is 24.9 in males and 24.6 in females, whereas the median is 18 for males and 17 for females. These figures all point to adolescence, during which a sudden burst of skeletal growth occurs, when the appearance of osteosarcoma is most likely (Dix *et al.*, 1983). Males are affected slightly more frequently than females. Data from the Memorial Sloan-Kettering Cancer Center in the New York City show that the proportion of male to female patients is 1.3:1. This can be accounted by the longer period of skeletal growth and the additional volume of bone produced in the male.

1.1.3. Clinical Features

The rate of tumour growth, degree of bone destruction and the severity of symptoms vary greatly among osteosarcoma patients. The earliest symptom is pain in the involved bone. The pain often irradiates to a neighbouring joint, which begins insidiously and intermittently. The pain then becomes severe and constant and could not be relieved by rest, immobilization, or ordinary analgesics. A palpable mass slowly develops, hindering joint motion. Swelling is minimal at first but becomes more prominent. The skin overlying the tumour is often shiny and stretched, with superficial veins showing prominence and dilatation. Local inflammatory signs and venous stasis are frequent in advanced lesions. Alkaline phosphatase activity increases either moderately or evidently in serum, which is related to the osteoblastic activity of the basic tumour cell (Huvos, 1991; Schajowicz, 1994). Usually, an open biopsy is performed for histological diagnosis.

The long bones of the extremities are the most common sites of osteosarcoma (Figure 1.2.). The femur is involved in 44% of all cases, the tibia in 17% and the humerus in 15%. The femoral involvement occurs most frequently in the distal part of the bone, especially in the metaphysis or diaphysis (Cook, 1996). In fact, 51% of osteosarcoma

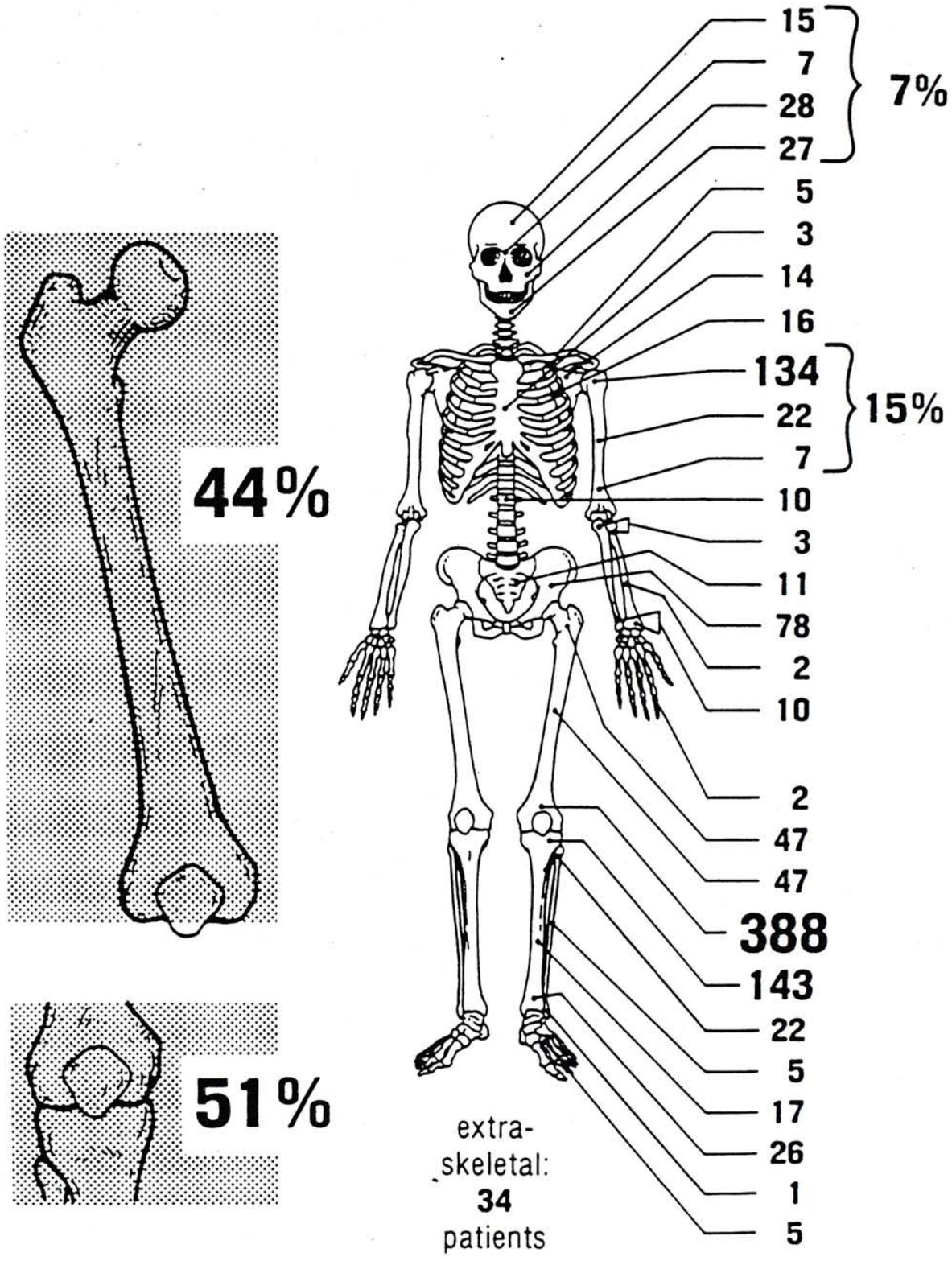


Figure 1.2. Skeletal distribution of osteosarcoma (Huvos, 1991). Most cases occured at the distal part of the femur. Half cases occurred around the knee region.

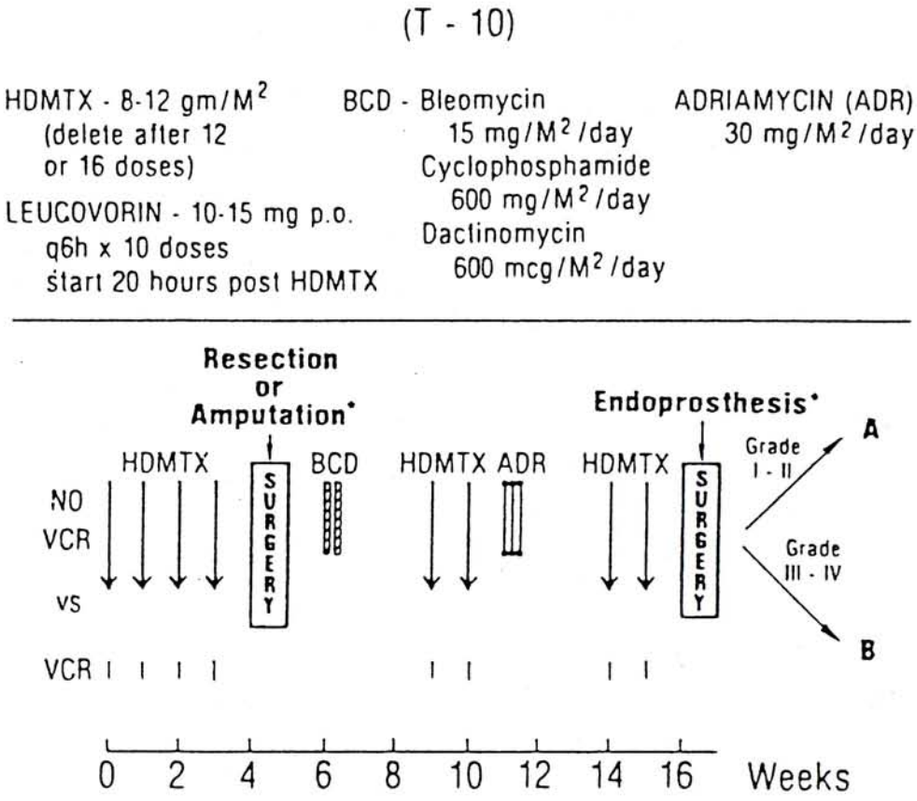
arises in the knee region. More than half of all osteosarcoma cases of the appendicular skeleton appear in patients who are under the age of 21. All other osseous or soft tissue sites are more common in skeletally mature older individuals.

1.1.4. Treatment

From experience, radiotherapy is not successful in either reliably controlling local recurrence or preventing the appearance of pulmonary metastases in osteosarcoma, even with doses that exceed normal tissue tolerance (de Moor, 1975). Complete surgical removal of the primary tumour, which can be an amputation or a limb salvage, is still the most reliable treatment of osteosarcoma. Although small pulmonary metastases may be successfully eliminated solely by aggressive chemotherapy, surgical removal of the primary tumour is still of critical importance (Douglass *et al.*, 1975).

Usually, surgical procedures are performed after pre-operative (neoadjuvant) chemotherapy. Previous reports have demonstrated that chemotherapy not only reduces the size of the primary tumour, but also entirely eradicates small tumours (Rosen *et al.*, 1974; Rosen *et al.*, 1975). The potential advantages of neoadjuvant chemotherapy are to enhance operability by shrinking the tumour and delineating tumour margins, which in turn decreases the likelihood of local recurrence. Neoadjuvant chemotherapy also serves as an accurate *in vivo* sensitivity test in gauging the susceptibility of the patient's sarcoma to the chemotherapeutic agents employed. The information help us plan the adjuvant chemotherapy to be given after operation.

At the Prince of Wales Hospital, the T-10 chemotherapy protocol is adopted for treating osteosarcoma patients (Figures 1.3. and 1.4.). This protocol was designed at the Memorial Sloan-Kettering Cancer Center in 1974 (Rosen & Nirenberg, 1982; Rosen *et al.*, 1982). Previously, the 5-year survival of patients undergoing surgery alone seldom exceeds 20%. After the introduction of the T-10 protocol, the figure boosts to more than 80%.



*Patients who are to undergo resection or amputation will have surgery at approximately four weeks. patients who are to undergo endoprosthetic replacement will have surgery at approximately 16 weeks.

Figure 1.3. Pre-operative chemotherapy for osteosarcoma (Rosen *et al.*, 1982). All patients receive the entire 16 weeks of induction chemotherapy, regardless of the time of surgery, before the selection of further adjuvant chemotherapy based upon the histologic response of the primary tumour.

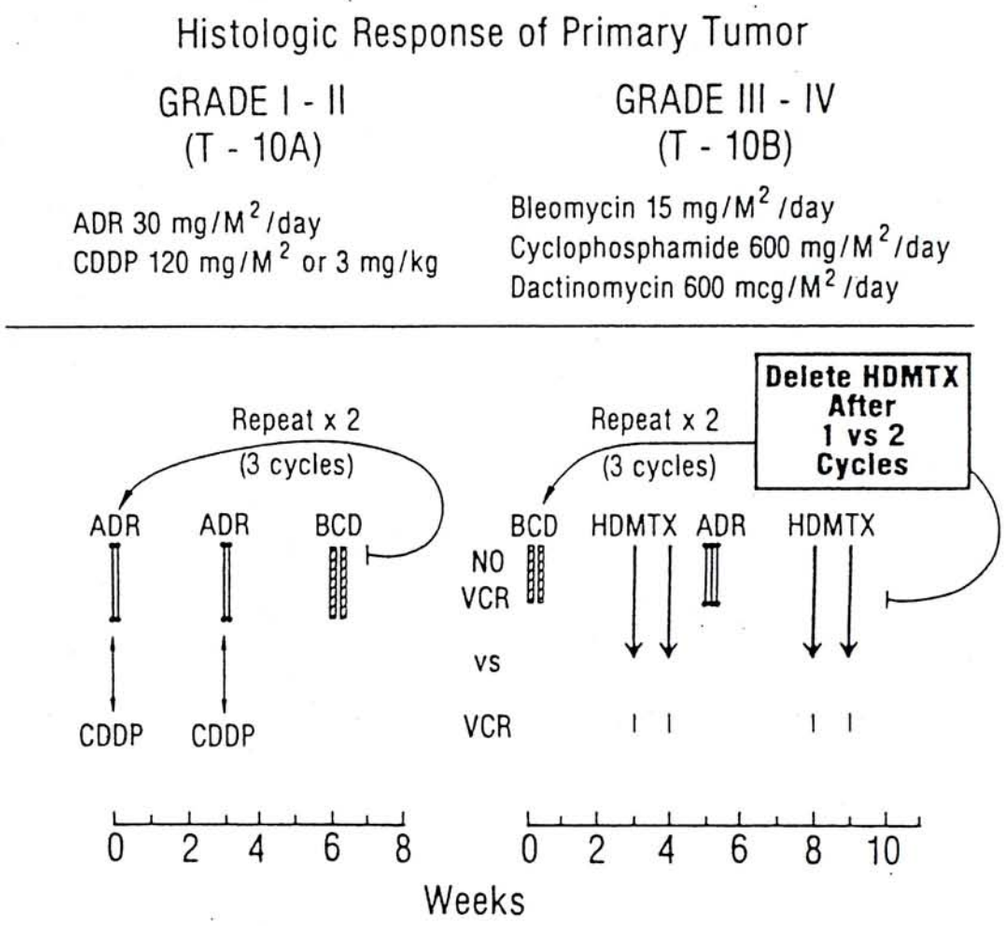


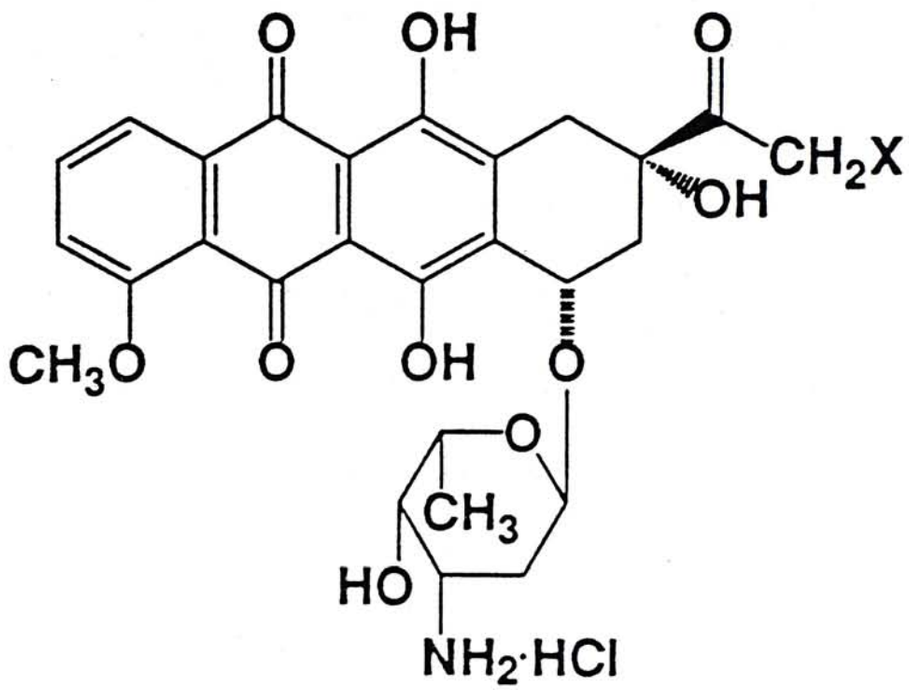
Figure 1.4. Maintenance chemotherapy for osteosarcoma (Rosen *et al.*, 1982). In regimen A, a combination of adriamycin and cisplatinum is used. In regimen B, high-dose methotrexate is the main agent in combination chemotherapy.

1.2. Adriamycin

Adriamycin (Doxorubicin) is an antitumour antibiotic first isolated in 1969 from *Streptomyces peucetius* (Arcamone *et al.*, 1969). It is a natural product which belongs to a group of chemicals known as the anthracyclines. The drug consists of a naphthacenequinone nucleus linked via a glycosidic bond at position 7 to the amino sugar, daunosamine (Figure 1.5.). It has a molecular formula of $C_{27}H_{29}NO_{11}$ and a molecular weight of 543.54. At room temperature it is in the form of orange-red colored crystals and gives an orange-red aqueous solution at neutral pH. Clinically it is used to treat acute leukaemia, lymphoma and solid tumours including osteosarcoma, and is considered as the antineoplastic drug possessing the broadest spectrum of antitumour activity.

1.2.1. Drug Action

There are several proposed mechanisms for the action of adriamycin. However, the precise mechanisms of cytotoxicity remain a mystery. Mechanistic studies indicated that the anthracycline drugs can intercalate into DNA, form reactive metabolites which are bound to cellular macromolecules, induce lipid peroxidation, inactivate various enzymes, and induce topoisomerase II-dependent DNA strand breaks. Some investigators thought that adriamycin mediates its action by binding to DNA and intercalating adjacent base pairs, thus preventing nucleic acid synthesis (de Fraine *et al.*, 1990). In another study, antioxidant vitamins were reduced and plasma thiobarbituric acid reactants were significantly increased in cancer patients at the end of adriamycin chemotherapy (Faure *et al.*, 1996). These suggest that drug-induced hydrogen peroxide and hydroxyl radical formation may play a role in the antineoplastic action of adriamycin (Doroshow, 1986). Adriamycin induced topoisomerase II-dependent DNA strand breaks may also lead to cell death (Potmesil *et al.*, 1987).



X = H, daunorubicin
X = OH, doxorubicin

Figure 1.5. Chemical structure of adriamycin (Acton, 1995). The molecule consists of a naphthacenequinone nucleus linked to daunosamine through a glycosidic bond at position 7.

1.2.2. Pharmacology

Adriamycin is administered intravenously over 3-5 minutes at a dosage of 60-75 mg/m^2 at 21-day intervals. The peak plasma drug concentration is 5mM (Muller *et al.*, 1997). The drug is eliminated by metabolic conversion to a variety of less active or inactive metabolites by liver enzymes. A major pathway is the conversion to the alcohol doxorubicinol which also has cytotoxic activity like its parent (Figure 1.6.). This reduction is catalyzed by cytoplasmic NADPH-dependent reductases. However, the reduction of adriamycin by these enzymes is slow, compared to another antitumour anthracycline daunorubicin. Therefore a few hours after administration, the principal circulating form of adriamycin is still the parent drug.

The major toxicity of adriamycin is in the heart. Since the anthracyclines are detoxified in the liver, patients with impaired hepatic function are at risk of severe toxicity and the dosage must be reduced as a result. At a total dose exceeding $550mg/m^2$ the patient is susceptible to rapidly progressive syndrome of congestive heart failure and cardiomyopathy. Other common side effects of the drug include alopecia, bone marrow depression, nausea and vomiting.

1.3. Multidrug Resistance

Chemotherapy is a major part in any treatment protocol to deal with osteosarcoma. Despite the fact that it increases the 5-year survival rate significantly, the emergence of drug resistance often confers a big problem to clinical oncologists. In most cases, tumour cells develop resistance to several drugs belonging to a particular structural class. However, certain antibiotics and plant alkaloids with very different structures and biochemical mechanisms of action can select for cells that are cross-resistant between drug classes. As a result, the cells may be resistant to some drugs which they have never been exposed to before. This phenomenon is known as multidrug resistance (MDR) (Pratt *et al.*, 1994).

MDR affects patients with a variety of hematological cancers and solid tumors

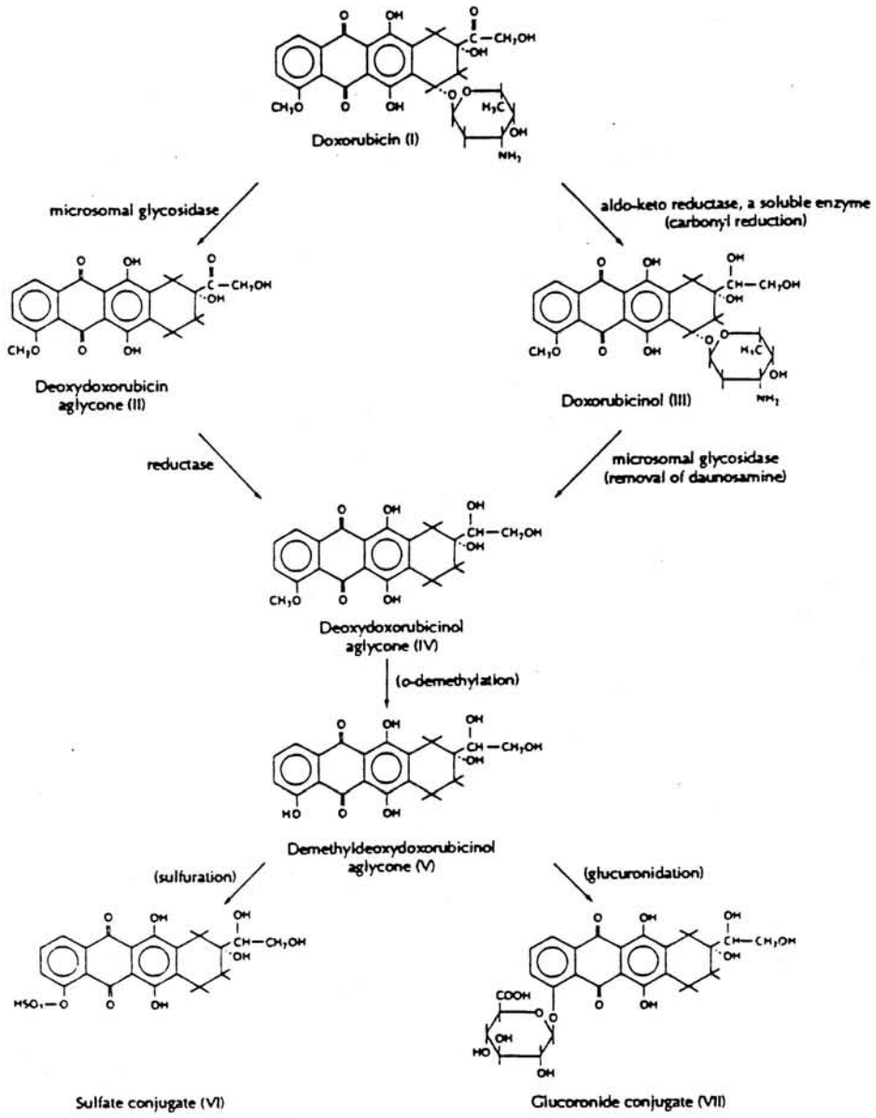


Figure 1.6. Major metabolic pathway for adriamycin in humans (Pratt *et al.*, 1994). The daunosamine residue is removed and the molecule is metabolised to give doxorubicinol sulphate or glucoronide conjugates.

including those of the breast, ovarian, lung and lower gastrointestinal tract. Resistant cells generally show an unresponsiveness to chemotherapeutic agents. In the presence of cytotoxic drugs, sensitive cells are killed, leaving resistant cells which continue to grow. This explains why MDR is usually observed after a course of chemotherapy.

There are several drug resistant mechanisms. The overexpression of membrane proteins, which control drug permeation, accelerates drug efflux, thus decreasing intracellular drug accumulation. For anthracycline antibiotics, free radicals are produced upon reduction. An enhanced antioxidative enzymes activities can scavenge excessive free radicals produced. A mutation in the topoisomerase II gene also prevents enzyme-dependent DNA strand breaks.

1.4. P-glycoprotein

In 1976, Juliano and Ling showed that a cell surface glycoprotein of 170kD was responsible for the drug resistance in Chinese hamster ovary cell mutants (Juliano & Ling, 1976). They designated it the P-glycoprotein (P-gp) (Figure 1.7.).

1.4.1. Nature

P-gp is a multidrug transporter which utilizes the energy of ATP to extrude a great variety of drugs from cells (Gottesman & Pastan, 1988). The protein is encoded by a small family of closely related genes, designated *mdr*, which includes two members in humans (*MDR*1, *MDR*2). The full length *MDR*1 cDNA encodes the whole P-gp molecule (Ueda *et al.*, 1986). P-gp is composed of 1280 amino acid residues, with a 140kD protein core and 30kD N-linked carbohydrate. The molecule is highly symmetrical, with 12 predicted transmembrane domains (Chen *et al.*, 1986; Richert *et al.*, 1988). Each half, known as a cassette, contains a very hydrophobic region and a hydrophilic region. It is thought that the two cassettes come together in the plasma membrane to form a channel for the energy-driven efflux of various drugs from the cell.

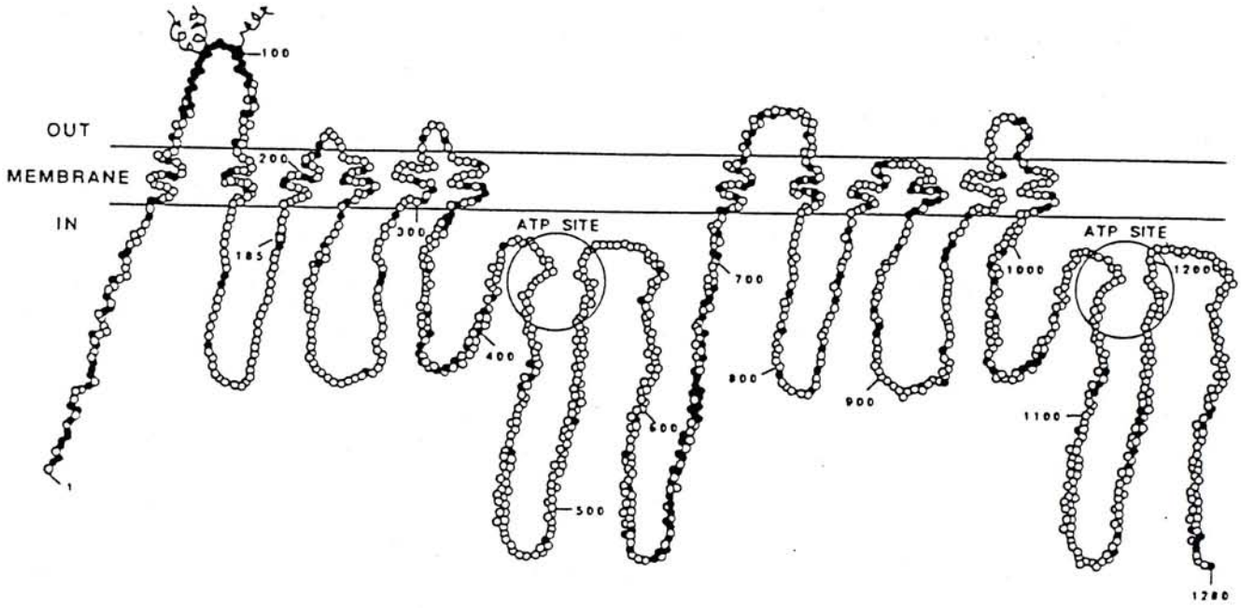


Figure 1.7. Human P-glycoprotein model (Gottesman & Pastan, 1988). The molecule is highly symmetrical. Each half contains 6 transmembrane domains and a ATP binding site.

Gros and Chen found that the P-gp in tumour cells bears strong homology to a class of well-studied bacterial transporter proteins named permeases (Gros *et al.*, 1986; Chen *et al.*, 1986). The ATP-binding proteins of all of these permeases share extensive homology and are highly homologous to the hydrophilic portions of the MDR proteins. The homology includes, and extends far beyond, the ATP-binding site, indicating a strongly conserved functional similarity.

1.4.2. Tissue Distribution

P-gp is found in both normal and neoplastic tissues (van der Valk *et al.*, 1990). It is localized to the luminal or apical surface of cells (Pratt *et al.*, 1994). Under normal conditions, it is distributed in the epithelium of various tracts and glands, for instance, the gastrointestinal tract and the salivary gland. It is also expressed in the endothelium of capillaries in various organs. Tumours arising from these normal tissues always have P-gp present. The presence of P-gp on cells with secretory or excretory functions suggests its physiological role in the removal of potentially toxic substances from different organs, or its responsibility for the secretion of endogenous molecules.

In the mineralizing region of human bones, P-gp is detected in growth plates (Mangham *et al.*, 1996). Immunohistochemical localization of P-gp to the perilacunar matrix in the hypertrophic zone of the growth plate, which gives the most intense staining, and to the osteoid matrix in the primary spongiosa, suggests the possibility of its association with matrix vesicles. Another point noteworthy is that P-gp contains two inwardly directed ATPase sites which may function as orthophosphate generators. It is believed that P-gp may play a role in chondrocyte hypertrophy.

1.4.3. Relation with MDR

P-gp mediated MDR is more commonly known as typical or classical MDR. P-gp contributes to drug resistance in about 50% of human cancers. In drug resistant bacteria or tumours cells, P-gp controls drug permeation by actively exporting intracellular drugs outside the cell. Overproduction of P-gp is thought to confer MDR

to tumour cells by decreasing the net intracellular accumulation of cytotoxic drugs. This overproduction has a genetic basis. It may be associated with gene amplification (de Bruijn *et al.*, 1986) or with an increased rate of transcription of the *MDR*1 gene (van der Bliek *et al.*, 1988). Cytogenetic analysis of P-gp-mediated multidrug-resistant sublines derived from human lung cancer cell line SW-1573 reveals translocations involving chromosome 7 at band q22. It is postulated that the 7q22 translocation is casually linked to a high level of P-gp expression (Nieuwint *et al.*, 1992).

1.5 Multidrug Resistance Protein

Several tumour cell lines selected *in vitro* for resistance against natural product anticancer drugs do not show increased P-gp expression. Instead they have amplification and overexertion of the multidrug resistance protein (MRP) gene (Zaman *et al.*, 1993; Barrand *et al.*, 1994).

The multidrug resistance protein (MRP) is first identified in 1990 (Marquardt *et al.*, 1990). It is a 190 kD membrane glycoprotein comprised of 1531 amino acids (Cole & Deelay, 1993). The protein is a member of the superfamily of ATP binding transport proteins. The MRP gene is located on chromosome 16 and overexpression of it is the consequence of chromosomal amplification and fragmentation. However, there is a possibility that the MRP gene overexpressed in MDR cell lines is simply coamplified while the real gene responsible for MDR resides with the MRP gene on the same amplicon (Berrand *et al*, 1994).

MRP has been detected in epithelia, muscle cells and macrophages (Flens *et al.*, 1996). Human lung tissues, both normal and neoplasmic, also show MRP expression (Giaccone *et al.*, 1996). The actual subcellular location of the protein in multidrug resistant cells is not clearly elucidated. The protein is present primarily in the endoplastic reticulum but lower levels are also contained in the plasma membrane fraction (Berrand *et al.*, 1994). In malignant tumours strong plasma membrane MRP staining was frequently observed, but in normal human tissues MRP staining was

predominantly cytoplasmatic (Flens *et al.*, 1996).

The clinical importance of MRP is still undecided. Although it has a minor sequence homology with P-glycoprotein (McGrath *et al.*, 1989), it is not clear whether the protein is involved directly in drug efflux. It is known that the level of expression of this protein in two series of non-small-cell lung tumour lines is closely associated with degree of resistance, with higher levels evident in the lines maintained at higher drug concentration (Barrand *et al.*, 1994). The protein may have an excretory function in protecting the organism against xenobiotics by exporting glutathione-conjugated endo- and xenobiotics (Müller *et al.*, 1994).

1.6. Reactive Oxygen Species

Cells generate energy aerobically by reducing molecular oxygen to water. During electron transfer, partially reduced oxygen species are produced. In fact, 1-2% of total oxygen consumption may be converted to superoxide anion radical ($\bullet O_2^-$), which is one of the reactive oxygen species (ROS). Other sources of ROS include radiation, toxic chemicals and drugs, e.g. adriamycin (Figure 1.8.). The superoxide anion radical may undergo spontaneous reduction to form hydrogen peroxide. This generated hydrogen peroxide is reduced in 3 ways: to be converted to less toxic substances by the cell, to be converted to hypochlorous acid by neutrophils and act as a bactericidal agent in phagocytic cells, or to be converted to the highly reactive hydroxyl radical spontaneously. The hydroxyl radical reacts instantaneously with any biological molecule, forming a more stable free radical (R&D, 1996).

1.6.1. Problems Arising from ROS

ROS are reactive species. They can react with all biological macromolecules. A radical can react with a macromolecule to generate a second radical, which in turn can react with a second macromolecule to continue the chain reaction (R&D, 1996). Among the more susceptible targets are the polyunsaturated fatty acids. The reaction

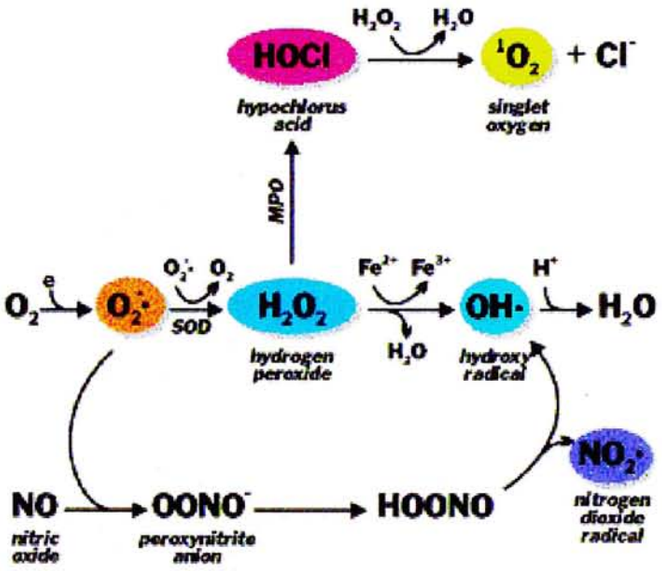


Figure 1.8. Generation of ROS (R&D, 1996). ROS can be generated by partial reduction of molecular oxygen or radiation, toxic chemicals and drugs, including adriamycin.

gives rise to many products, mostly alkanals and alkenals. ROS also modify the structure and function of proteins. They cause group additions, cross-links or fragmentation of proteins. Similarly, modification of nucleotide bases results in single-strand breaks and cross-links in DNA.

1.6.2. Oxidative Stress and Diseases

It is now believed that many diseases are associated with an abnormal metabolism of ROS. Atherosclerosis and ischaemia/reperfusion after myocardial infarction (Omar *et al.*, 1991) are examples of oxidative-stress-associated cardiovascular problems. Neurological diseases, like, Alzheimer and Parkinson diseases, and musculoskeletal disorders, such as Duchenne muscular dystrophy (Hunter & Mohamed, 1986) and multiple sclerosis (Hunter *et al.*, 1985) are also associated with ROS.

Free radicals also initiate carcinogenesis by activating carcinogens (Gonzales *et al.*, 1984; Trush & Kensler, 1991). Most chemicals must first undergo metabolic activation to a biologically reactive intermediate to contribute to tumour promotion. This activation could be brought about by an oxidant generated by enzyme catalysis, or by the attack from oxidized biomolecules.

1.6.3. Defense System

ROS cause damage to cellular components. In order to minimize their damage, mammalian cells possess elaborate defense mechanisms to detoxify ROS. The key metabolic steps are the dismutation of superoxide to hydrogen peroxide and oxygen by superoxide dismutase, and the conversion of hydrogen peroxide to water by catalase and glutathione peroxidase (McCord & Fridovich, 1969; Maddipati & Marnett, 1987) (Figure 1.9.). Other chemical antioxidants (e.g. vitamins C and E) terminate the chain reaction of radical damage by capturing the free radicals.

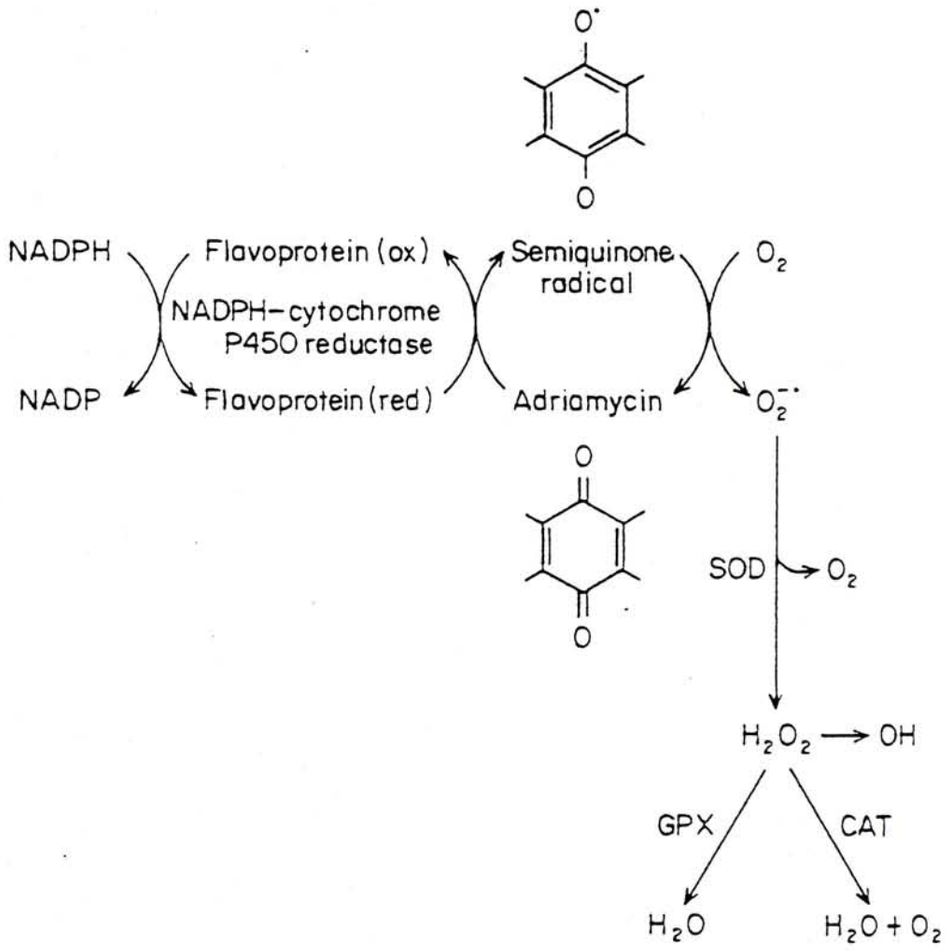


Figure 1.9. Adriamycin-induced oxyradicals formation and their removal by cellular antioxidative enzymes (Meijer *et al.*, 1990). The superoxide radical is converted into hydrogen peroxide by superoxide dismutase and then to water and oxygen by catalase or glutathione peroxidase.

1.6.4. Antioxidative Enzymes

Superoxide dismutase (SOD) (EC 1.15.1.1) exists in two forms: Cu, Zn SOD which is found primarily in the cytoplasm, and the Mn SOD which is found predominantly in mitochondria (Powers *et al.*, 1978). The molecular weight of SOD is approximately 31kD. Both isoforms catalyze the dismutation of superoxide ions into hydrogen peroxide and oxygen (Fridovich, 1986).

$$2\,O_2^{\cdot-} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

Catalase (EC 1.11.1.6) is a 240kD heme-containing tetrameric enzyme found mainly in peroxisomes and mitochondria (Aebi & Wyss, 1978; Chance *et al.*, 1979). It catalyzes the conversion of hydrogen peroxide to oxygen and water:

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Glutathione peroxidase (GPx) (EC 1.11.1.9) also exists in two forms: selenium dependent and selenium independent isoenzymes. The selenium-containing GPx is a tetrameric enzyme with molecular weight 84kD. It catalyzes the reduction of alkyl hydroperoxides to the corresponding alcohol by coupling to the glutathione redox cycle:

$$ROOH + 2\,GSH \longrightarrow ROH + GSSG + H_2O$$

The oxidized glutathione (GSSG) is reduced back to GSH by the dimeric flavoprotein glutathione reductase, at the expense of NADPH: (Staal *et al.*, 1969)

$$GSSG + NADPH + H^+ \longrightarrow 2\,GSH + NADP^+$$

1.6.5. Relation with MDR

Human cells are capable of activating adriamycin to its semiquinone status to generate secondary oxyradicals, except in adriamycin-resistant cells. Evidence shows that enzymes that detoxify superoxide and hydrogen peroxide significantly inhibit adriamycin-induced hydroxyl radical formation. This reduction in free radical formation in the nuclei and mitochondria of resistant cells may be significant in the protection of adriamycin-induced cell killing (Sinha *et al.*, 1987; Sinha *et al.*, 1989). As a result, increased activities of antioxidative enzymes are thought to be one of the factors contributing to MDR for anthracycline drugs.

1.7. Topoisomerase II

During replication, helical DNA must first be unwound to relax the coil. The molecule's topology is controlled by two types of topoisomerases. Type I topoisomerase functions by creating transient single-strand breaks in DNA while type II topoisomerase modulates DNA topology by binding covalently with DNA and thereby generating a transient double-strand break in one double helix through which a second double helix is passed (Mirski & Cole, 1997). Topoisomerase II is a homodimer. Topo IIα has two 170kD subunits and topo IIβ has two 180kD subunits. The two isoforms are encoded by distinct genes (Chung *et al.*, 1989).

If the topoisomerase II activity in a cell is reduced, fewer cleaved complexes can be formed and drug resistance may be the result (Figure 1.10.). The amount of topoisomerase II present in a cell may be regulated by protein turnover as well as by transcription, which is tightly controlled. Post-translational modification of the polypeptide or a mutation of the topoisomerase II gene may alter the enzyme's activity (Potmesil *et al.*, 1987; de Isabella *et al.*, 1991). However, the MDR associated with an altered topoisomerase II activity is a well-defined system of drug resistance

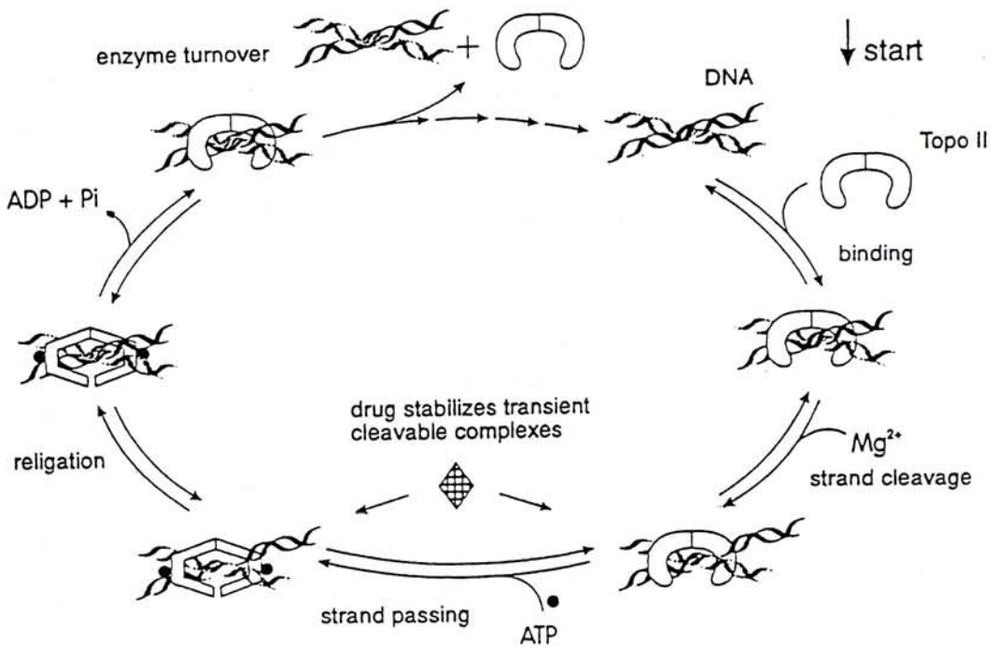


Figure 1.10. Catalytic cycle of topoisomerase II and generation of drug-stabilized cleavable complexes (Mirski & Cole, 1997). The enzyme catalyses the unwinding of DNA by creating a transient double-strand break Some chemotherapeutic drugs appear to stabilize the transient DNA-topoisomerase II cleavable complexes by either increasing the forward rate of DNA cleavage or inhibiting DNA religation.

only in tissue culture models for which a clinical correlation has not been found yet due to limited clinical studies of the enzyme (Booser, 1997)

1.8. Methods to Detect MDR

Several methods have been developed to detect MDR on clinical specimens and cultured cells. The most widely adopted traditional method is the P-gp immunohistochemistry. The adriamycin binding assay is recently developed as an alternative. Both methods can be applied on clinical specimens as well as on cultured cells.

1.8.1. P-glycoprotein Immunohistochemistry

Immunohistochemistry is a special technique developed for the detection of cell surface and intracellular antigens in slices of fresh or fixed human (or animal) tissues by using antibodies. After incubation with a primary antibody reactive against a specific antigenic epitope, the tissues are treated with a second step antibody, raised in a different species, that binds the constant region of the primary antibody. This secondary antibody is conjugated to an enzymatic agent, such as biotin-avidin peroxidase complex, in several additional incubation steps. Finally, a chromogen (e.g. diaminobenzidine) is added, producing a reddish or brownish pigment. This color may be visualized on a glass slide using a light microscope, or be quantitated by optical density, using computer assisted image analyzer. Immunohistochemistry is the only method currently available for detecting protein expression in archival patient tissues stored in paraffin blocks.

P-gp immunohistochemical studies on various cancers show that P-gp expression is associated with a poor prognosis in breast cancers (Schneider & Romero, 1995). Ovarian adenocarcinoma patients with P-gp-positive post-chemotherapy neoplasms are at three times greater risk of dying within 2 years than their counterparts with P-gp-negative tumours (Khalifa *et al.*, 1997). P-gp positive patients are also at

significantly greater risk of disease recurrence (Gregorcyk *et al.*, 1996). In squamous cell carcinomas, only tumour cells at the invasion front express P-gp (Beer *et al.*, 1996). This makes some researchers believe that P-gp expression may enhance the invasive potential of tumour cells. Laboratory analysis confirms that the protein is found to be expressed more frequently in metastatic than in primary tumours. Its expression seems to be a marker of a more malignant phenotype (Linn *et al.*, 1995).

On the other hand, some claim that there is a strong reverse relation between P-gp expression and proliferative activity in endometrial adenocarcinomas (Kodama *et al.*, 1995). For instance, Nakagawa *et al.* showed that P-gp mediated multi-drug resistance might be induced after chemotherapy of bladder tumors. However, the presence of P-gp before chemotherapy does not predict clinical outcome (Nakagawa *et al.*, 1997).

1.8.2. Adriamycin Binding Assay

Adriamycin is a natural fluorochrome. Intracellular adriamycin accumulation may be monitored by direct microscopic observation. Different patterns of fluorescence have been observed in living sensitive and resistant cells, and responsiveness to adriamycin has been associated with selective binding to the nucleus. The adriamycin binding assay (ABA) was developed in 1992 as a means to assess the chemosensitivity of clinical osteosarcoma specimens (Gebhardt *et al.*, 1994). By observing the binding pattern of adriamycin in the tumour cells, the sensitivity of the tumour can be determined. It is shown that low levels of resistance *in vitro*, being not detectable by increased levels of expression of P-gp, can be revealed by the assay (Baldini *et al.*, 1992). However, its applicability on clinical specimens has not been demonstrated, nor has there been any report about the correlation between patients' ABA results and their clinical outcome.

1.9. Aims of Study

Early detection of multidrug resistance (MDR) in osteosarcoma patients may provide

clinicians with drug response and prognostic information. For drug resistant patients, suitable alternative treatment can be planned. Since classical MDR is mediated by P-gp, an evaluation of P-gp expression in biopsy samples may show intrinsic resistance. P-gp immunohistochemistry offers a way to measure the amount of P-gp expressed in a cell. However, the correlation of P-gp with clinical outcome remains controversial. Some authors reported that P-gp overexpression was associated with a poor prognosis, metastasis or even recurrence (Schneider & Romero, 1995; Linn *et al.*, 1995; Gregorcyk *et al.*, 1996). Others found no correlation between P-gp expression and patient outcome (Nakagawa *et al.*, 1997; Nezasa *et al.*, 1997). On the other hand, the adriamycin binding assay has been proved to be a good method to determine *in vitro* adriamycin resistance recently (Baldini *et al.*, 1992), though its clinical application has not been established. In this study, we tested both methods on osteosarcoma samples to assess their correlation with tumour necrosis.

Anticancer drugs have to gain access to tumour cells before they can exert their cytotoxic effects. An investigation on the effect of adriamycin on osteosarcoma cells help us to have a better understanding of the basis and the initial development of MDR. This may facilitate the development of new protocols to combat MDR. As mentioned above, anticancer quinones may kill tumour cells by multiple mechanisms and hydrogen-peroxide mediated events can predominate at lower drug concentrations (Doroshow, 1986). On the other hand, Lee and his co-workers reported that glutathione peroxidase activity was elevated during the initial development of adriamycin resistance (Lee *et al.*, 1996b). Since both catalase and GPx catalyze the decomposition of hydrogen peroxide, therefore we treated osteosarcoma cells with a low dosage of adriamycin to evaluate their catalase and GPx activities.

2. MATERIALS AND METHODS

2.1. Clinical Study

Clinical osteosarcoma samples were subjected to the adriamycin binding assay and P-gp immunohistochemistry. Post-chemotherapy osteosarcoma specimens removed by surgery were sent to a pathologist for tumour necrosis assessment.

2.1.1. Patients Recruitment

Osteosarcoma samples were obtained from patients admitted to the Department of Orthopaedics and Traumatology of the Prince of Wales Hospital. Patients were randomly chosen, regardless of their sex, age and the site involved. Their consensus was obtained before taking biopsy and surgical removal of the tumour. Biopsy samples were obtained before chemotherapy to confirm diagnosis. Surgical removal of tumour was performed after the patients had received one or more courses of chemotherapy. Both types of samples were subjected to the ABA and P-gp immunohistochemistry. Tumour necrosis was assessed by a pathologist only for samples taken after chemotherapy.

2.1.2. Adriamycin Binding Assay

Biopsy of osteosarcoma was taken from patients in the operating theatre. The sample was briefly washed with phosphate buffered saline (PBS) and sterilized after being immersed into 10% penicillin-streptomycin-neomycin (PSN) antibiotic mixture (15640-055, Gibco, Maryland) for 1-2 minutes. After a brief mechanical dissociation, the sample was treated with collagenase (C-6885, Sigma, St. Louis) at 37°C for at least 1 hour until adequate cell suspension was obtained. Tissue debris was removed by filtration through a 100μm membrane filter (Whatman, Massachusetts). After

adding more culture medium to dilute the collagenase, the cell suspension was centrifuged at 1,500g for 5 minutes. The supernatant was aspired away and the cells were resuspended in fresh Iscove's Modified Dulbecco's Medium (IMDM) (I-7633, Sigma, St. Louis). The number of cells released was determined using a haemocytometer. Cell viability was determined using the trypan blue exclusion method.

2×10^4 cells were resuspended in 1ml IMDM. 5µl adriamycin (Farmitalia Carlo Erba, Milan) solution was added. After incubating at 37°C for 30 minutes under continuous motion, 2ml fluorescein diacetate (F-7378, Sigma, St. Louis) solution was added and the cell suspension was further incubated for 10 minutes in the dark. 1ml PBS was then added to the suspension. After centrifugation at 1,500g for 5 minutes, the cell pellet was resuspended in 50µl medium and the cells were examined under a fluorescence microscope (DMR photomicrography system, Leica, Wetzlar) with blue and green filters.

Living cells were first identified using fluorescein diacetate fluorescence. Under blue excitation (blue filter), they showed green fluorescence, while dead cells did not (Figure 2.1.). Adriamycin sensitivity was then recorded under red excitation (green filter). Sensitive cells showed strong nuclear and weak cytoplasmic fluorescence. Resistant cells had weak diffuse cytoplasmic fluorescence without any distinctive intracellular binding (Figure 2.2.). The percentage of sensitive cells was calculated based on 300 living cells. According to Baldini, samples with 50% or more sensitive cells were regarded as sensitive whereas those with less than 50% sensitive cells were regarded as resistant.

2.1.3. P-glycoprotein Immunohistochemistry

P-gp immunohistochemistry was performed on osteosarcoma samples preserved in paraffin blocks. Both pre-chemotherapy and post-chemotherapy samples were used.

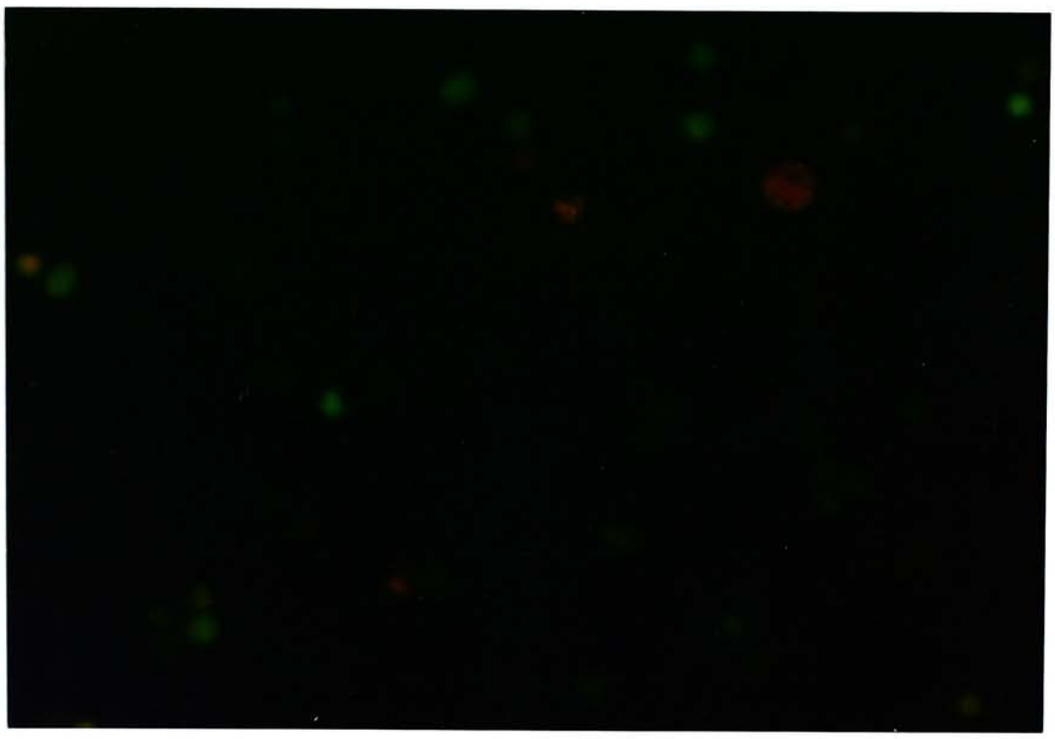


Figure 2.1. A fluorescence micrograph showing SaOS-2 cells stained with fluorescein diacetate (200x). Under blue excitation, living cells are stained green while dead cells are not.

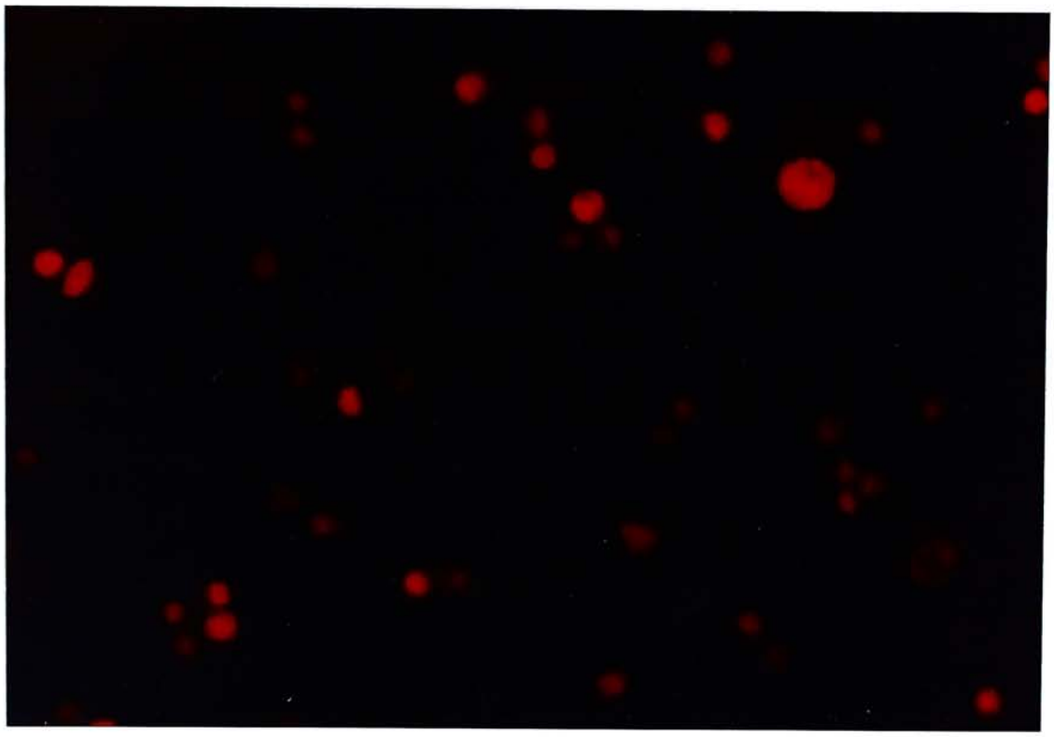


Figure 2.2. A fluorescence micrograph showing adriamycin sensitive and resistant cells (200x). Under red excitation, sensitive cells show strong nuclear and weak cytoplasmic staining while resistant cells have weak staining without any distinctive intracellular binding.

2.1.3.1. Sample and Control Preparation

Tumour samples from osteosarcoma patients were decalcified with 10% formic acid/formalin and fixed with 10% neutral buffered formalin for 2-3 days. They were dehydrated by upgraded alcohol series and embedded in paraffin. 5μm thick sections were cut using a microtome (Reichert-Jung 1130 Biocut, Leica, Nussloch).

Human cervical carcinoma cell line KB-V1 served as the positive control for P-gp immunohistochemistry. The cell line is well-known for its high expression of P-gp on its surface (Cornwell *et al.*, 1986). In order to mimic the tissue processing conditions that could influence immunoreactivity, a KB-V1 cell pellet was prepared. Preparation of cell culture cell blocks was described by Bratthauer (Bratthauer, 1994). Briefly, cells were cultured in a culture flask to confluence and then removed by the addition of Trypsin-EDTA (25200-056, Gibco, Maryland) solution. They were washed twice with PBS. To form a pellet, cells were centrifuged at 1,500g for 10 minutes. Next, PBS was decanted and 10% neutral buffered formalin overlaid the pellet without causing disturbance. The pellet was fixed overnight at 4°C. Then the fixative was decanted and Milli-Q water overlaid the pellet. Carefully undermining the pellet with the needle of a syringe of Milli-Q water along the wall of the tube, the pellet was dislodged by rapidly expressing the syringe. To take out the pellet, it was swirled in Milli-Q water and quickly poured into a beaker. The Milli-Q water was removed and 70% alcohol was added to cover the pellet. Then the pellet was processed as solid tissues in a processor (Reichert-Jung Histokinette 2000, Leica, Nussloch). It was dehydrated by up-graded alcohol and embedded into paraffin. Then 5μm thick sections were cut using a microtome (Reichert-Jung 1130 Biocut, Leica, Nussloch).

2.1.3.2. Immunohistochemical Procedures

Osteosarcoma sections were deparaffinized in xylene and rehydrated in down-graded alcohol. After being rinsed in PBS, the tissue sections were briefly digested by trypsin (T-8253, Sigma, St. Louis). Endogenous peroxidase activity was quenched by

exposing tissue sections to 3% H_2O_2 solution (18304, RDH, Seelze) for 10 minutes. Non-specific antibody binding was reduced by the addition of 1% bovine serum albumin (BSA) (A-9647, Sigma, St. Louis) to tissue sections for 20 minutes. The slides were incubated with primary antibody (SC-1517, Santa Cruz, California) at room temperature for 1 hour, and then secondary antibody (A-3540, Sigma, St. Louis) for 30 minutes. Colour was developed for 10 minutes in chromogen substrate solution. Haematoxylin was used as counterstain. All steps were repeated with the primary antibody substituted by the blocking solution on the negative control slide. Immunohistochemistry results were analyzed under a light microscope. P-gp positive cells were stained brown while negative cells did not (Figures 2.3. and 2.4.).

2.1.4. Tumour Necrosis Assessment

The effect of chemotherapy on primary sarcomas was assessed by a pathologist (Huvos, 1991). Osteosarcoma samples were cut into small blocks. The blocks were processed and embedded in paraffin for sectioning. Grades of response to pre-operative chemotherapy were assigned following histological analysis of approximately 20-60 tissue sections (Table 2.1.). A grade I or II is considered no effect or a partial effect, which is regarded a "poor necrosis". A grade III or IV is considered a complete or near-complete effect, which is regarded a "good necrosis" (Figures 2.5. and 2.6.).

2.2. Effect of Adriamycin on Osteosarcoma Cells

To study the effect of adriamycin on osteosarcoma cells, an *in vitro* model was established.

2.2.1. Establishment of Adriamycin Adapted Osteosarcoma Cells

Human SaOS-2 cell line (HTB-85) was purchased from the American Type Culture

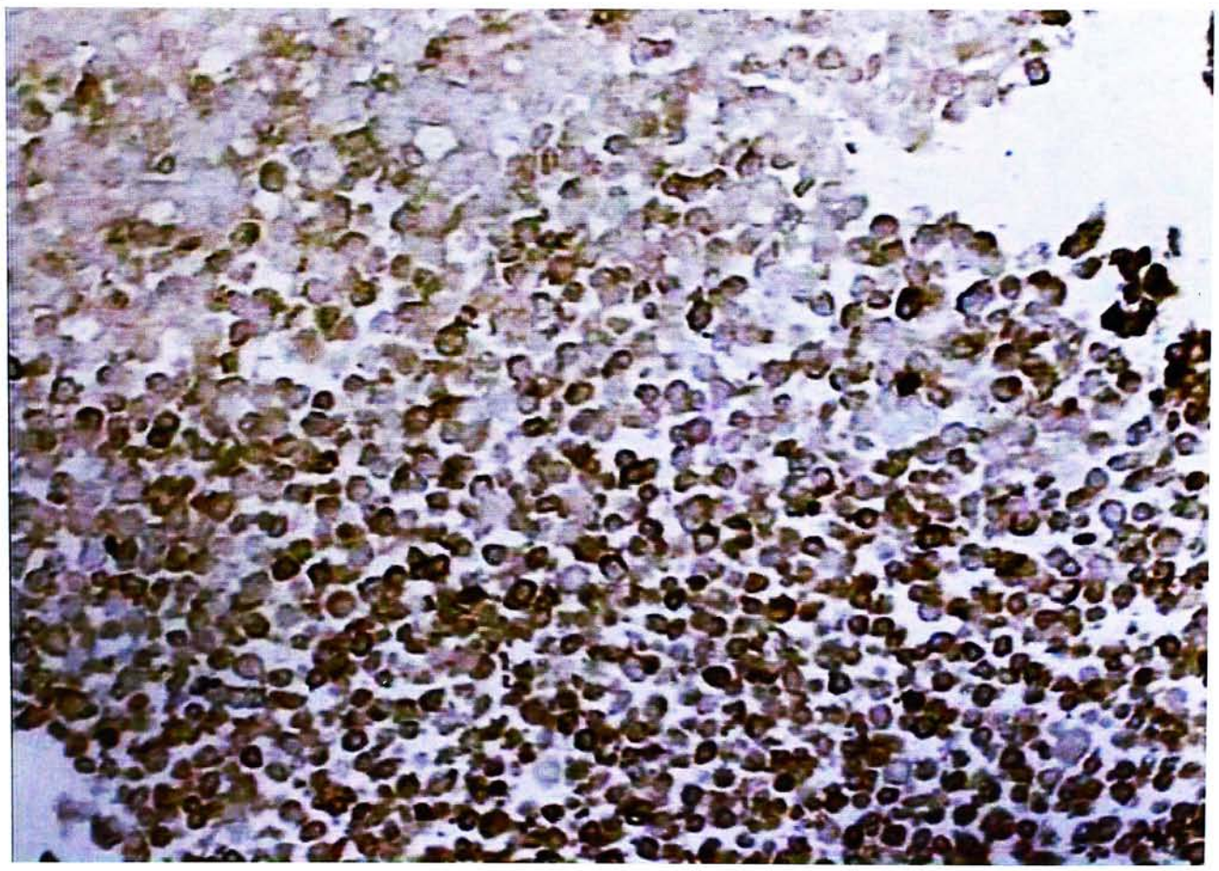


Figure 2.3. A light micrograph showing positive P-glycoprotein immunostaining of paraffin embedded KB-V1 cells using diaminobezidine as substrate (200x).

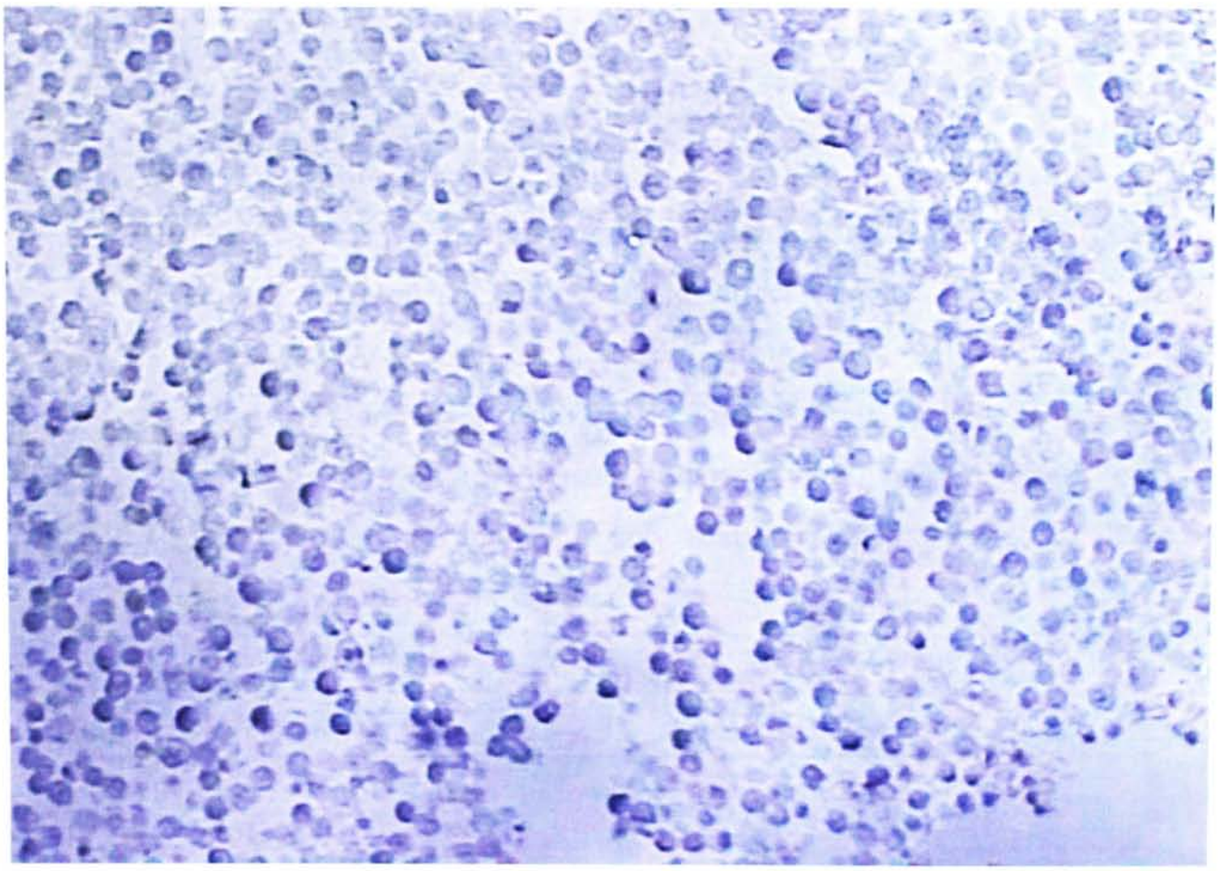


Figure 2.4. A light micrograph showing negative P-glycoprotein immunostaining of paraffin embedded KB-V1 cells using diaminobezidine as substrate (200x).

Grade	*Effects*
I	Little or none.
II	Areas of acellular tumour osteoid, necrotic, and/or fibrotic material attributable to the effect of chemotherapy, with other areas of histologically viable tumour.
III	Predominant areas of acellular tumour osteoid, necrotic, and/or fibrotic material attributable to the effect of chemotherapy, with only scattered foci of histologically viable tumour cells.
IV	No histologic evidence of viable tumour within the specimen.

Table 2.1. Histologic grading of preoperative chemotherapy effects on primary osteosarcoma (Huvos, 1991). Grades I and II correspond to poor response. Grades III and IV correspond to good response.

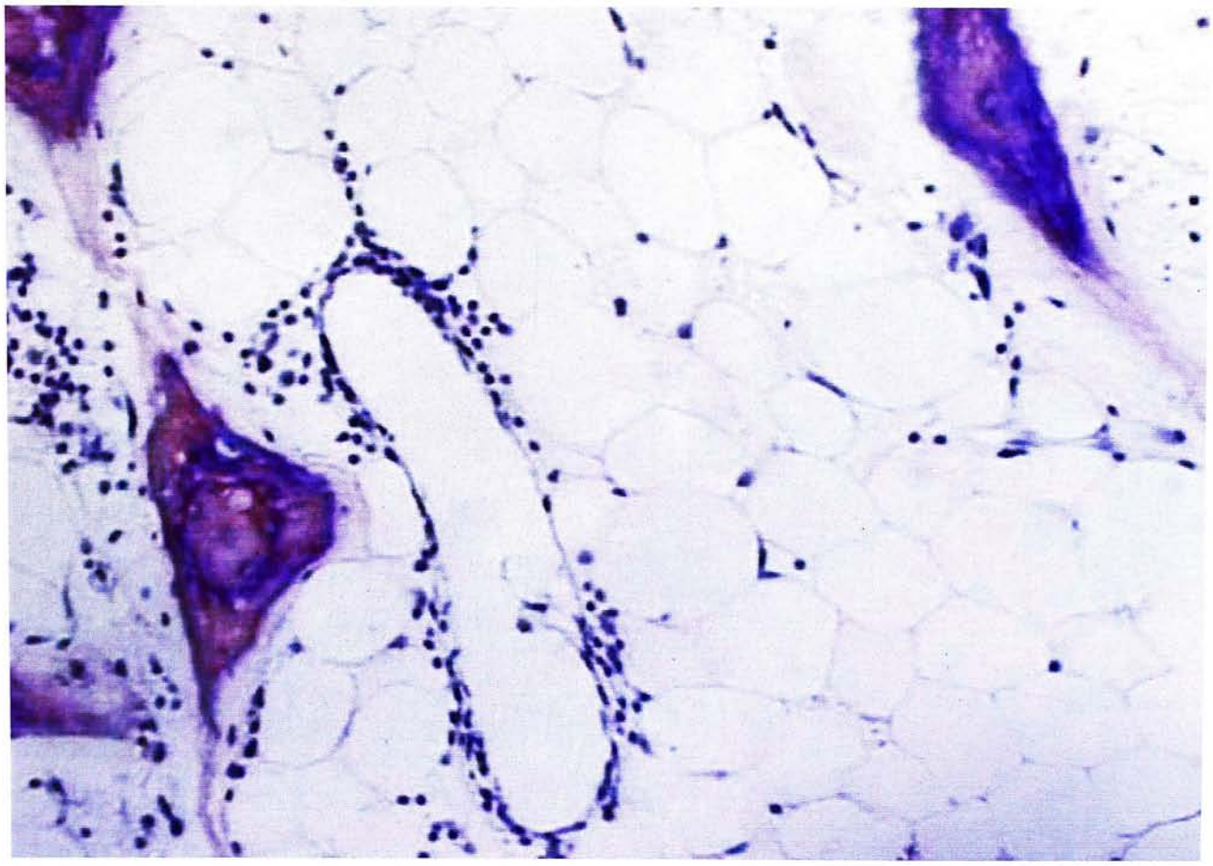


Figure 2.5. A light micrograph showing post-chemotherapy osteosarcoma taken from patient YCY stained with hematoxylin and eosin (200x). Good necrosis was observed as most tumour cells were killed by chemotherapeutic agents, leaving their nuclei behind.

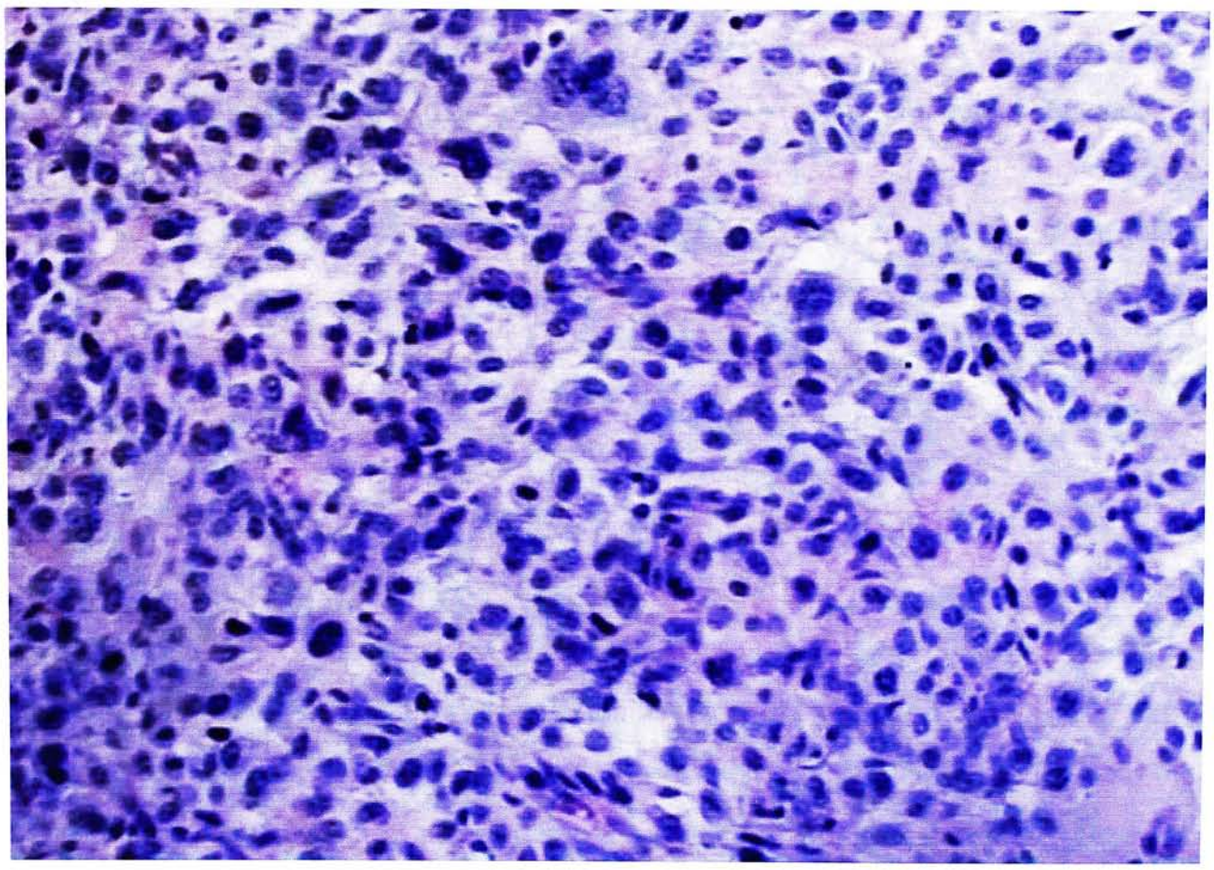


Figure 2.6. A light micrograph showing post-chemotherapy osteosarcoma taken from patient SHL stained with hematoxylin and eosin (200x). Poor necrosis was observed as most tumour cells reamined viable.

Collection. It is a primary osteogenic sarcoma cell line derived from an 11 years old Caucasian female. The cells grow as a monolayer with an epithelial-like morphology (Figure 2.7.).

2.2.1.1. Maintenance and Subculture of SaOS-2 Cell Line

SaOS-2 cells were cultured in 150cm^2 culture flasks (430824, Corning, Massachusetts). The cells were kept at 37°C, 5% CO_2 and 100% humidity. The culture medium was changed every 2-3 days. When confluence was reached, the medium in the culture flask was discarded and the cells were washed with PBS. Trypsin-EDTA solution (25200-056, Gibco, Maryland) was added to the monolayer and the culture flask was incubated at 37°C until the cells were well rounded and began to detach. Cells were dislodged by sharply tapping the culture flask against the palm of hand. The action of trypsin was stopped by adding several milliliters of medium containing fetal bovine serum (FBS) to the cell suspension. The released cells were then transferred to a sterile centrifuge tube. The culture flask was rinsed with PBS and the solution pooled together. The cells were centrifuged at 1,500g for 10 minutes. After the supernatant was removed, the cell pellet was resuspended in fresh medium. Cell viability was determined under haemocytometer by the trypan blue exclusion method. 10µl cell suspension was mixed with 10µl trypan blue solution and then placed in a haemocytometer. Under a light microscope, dead cells were stained blue while viable cells were not. The numbers of dead and viable cells were then counted respectively. Only the number of cells between 200 to 500 was accepted. SaOS-2 cell line was maintained by seeding 1 x 10^6 cells to another 150cm^2 culture flask containing the supplemented medium.

2.2.1.2. Storage of Cell Line

To store SaOS-2 culture, cells grown to confluence were trypsinized and centrifuged at 1,500g for 10 minutes. The cell pellet was resuspended in supplemented medium at a concentration of 1 x 10^7 cells/ml. 1ml cell suspension was dispensed aseptically into

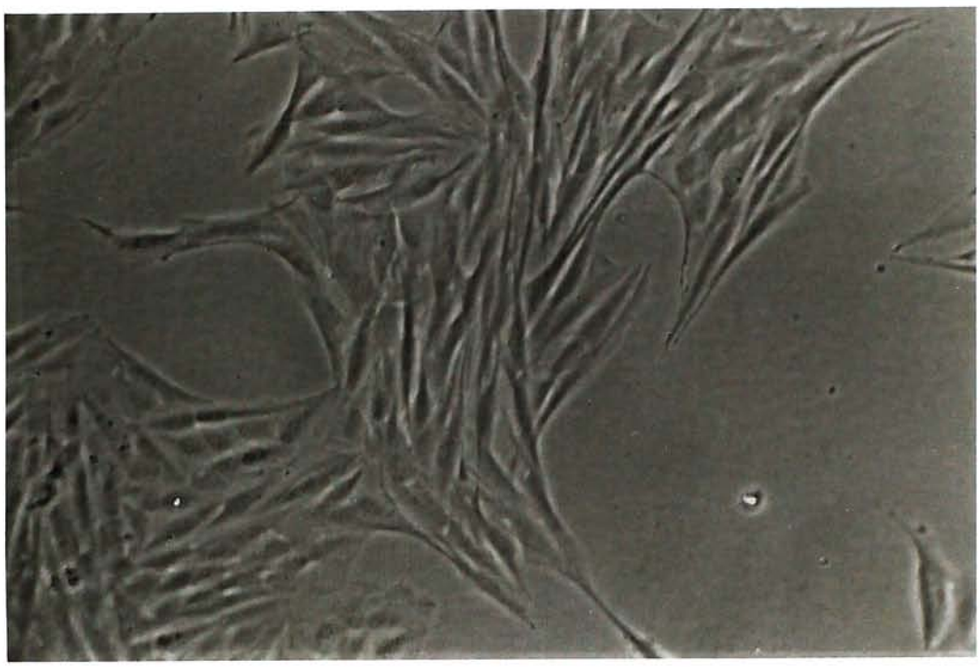


Figure 2.7. A phase contrast inverted light micrograph showing SaOS-2 cells in culture for 2 days (100x). The cells grow with an epithelial-like morphology.

a cryogenic vial and 50μl dimethylsulphoxide (D-5879, Sigma, St. Louis) was added. The vial was frozen at -20°C overnight and then stored in liquid nitrogen.

2.2.1.3. Adriamycin Treatment

SaOS-2 cells were stepwisely exposed to 20ng/ml and 40ng/ml adriamycin (Farmitalia Carlo Erba, Milan) (Serra *et al.*, 1993). 2 days after subculture, adriamycin was added to a final concentration of 20ng/ml. Cells were grown in this concentration of adriamycin for 2 weeks, and then stepped up to 40ng/ml. The cells were continuously cultured in the presence of adriamycin for at least 2 weeks before experiments.

2.2.2. KB-V1 Cell Culture

KB-V1 cell line is a gift from the City University of Hong Kong. The human epidermoid carcinoma cell line is a Hela variant. It is derived *in vitro* by a stepwise selection in the presence of vinblastine.

Cell line maintenance and subculture procedures were the same as for SaOS-2 cell line. To maintain its expression of P-gp, vinblastine solution was added into the culture medium the following day after each subculture to a final concentration of 1μg/ml.

2.2.3. Adriamycin Binding Asasy

The assay procedures were the same as for clinical samples except that cell releasing steps were omitted.

2.2.4. P-glycoprotein Immunohistochemistry

For immunostaining of cultured SaOS-2 cells, cytospin preparation of KB-V1 served as the positive control. Both types of cells were harvested from cell culture as described. Cells were diluted to a concentration of 20,000 cells/ml. 1ml cell suspension was spun down on a glass slide (Cytospin, Shandon Lipshaw, Pennsylvania) at 1,000 rpm for 10 minutes. The slide was then fixed for 10 minutes in cold acetone as suggested by other investigators (Broxterman *et al.*, 1989; Beck *et al.*, 1996). Each cell patch contains 20,000 cells in 5mm diameter. Immunohistochemical procedures were the same as for clinical samples.

2.2.5. Thymidine Incorporation Assay

DNA synthesis is an essential step in cell proliferation. Adenosine, thymidine, guanine and cytosine are the four bases composing a DNA molecule. Of these four bases, only thymidine is unique in a DNA. The thymidine incorporation assay makes use of this property to measure the rate of DNA synthesis (Stenberg, 1986). Tritiated thymidine is added into the culture medium for cells to take up. The radioactivity emitted from a cell's DNA is an index of its rate of DNA synthesis, which reflects its proliferation rate.

2.2.5.1. Assay Procedures

Osteosarcoma cells obtained from culture were diluted in supplemented medium. 2 x 10^4 cells were seeded into each well of a 24-well plate (430262, Corning, Massachusetts). The cells were incubated at 37°C, 5% CO_2 and 100% humidity overnight so that they can to adhere to the bottom of the plate. The next day, medium in each well was aspired. 300µl (6µCi) freshly prepared labeled thymidine (TRK328, Amersham, Uppsala) solution was added. Then, the cells were incubated at 37°C, 5% CO_2 for 4 hours. At the end of the incubation, the labeled thymidine solution was aspired and the wells were washed with 1ml PBS to remove any unincorporated

labeled thymidine.

To lyse the cells, 250µl 0.25M NaOH was added into each well and after 15 minutes, the wells were rinsed with another 250µl NaOH. The cell lysate was transferred into a centrifuge tube. 500µl 0.25M HCl was added to neutralize the alkalinity. To precipitate DNA, 1ml 0.25mM Hepes with BSA solution was added into each tube, followed by 0.5ml 10M perchloric acid (524, Merck, Darmstadt). The solution was refrigerated at 4°C for another 30 minutes.

After refrigeration, the tubes were centrifuged at 13,000 rpm at 4°C for 30 minute to pellet the BSA which carried the DNA. The supernatant was discarded and the pellet was resuspended in 0.5ml 0.25M NaOH. The solution was transferred to a counting vial and mixed with 4ml scintillation fluid. The number of counts per minute was recorded by a liquid scintillation counter (LS 3801, Beckman, California). Background radioactivity was measured in the same way by substituting the sample with 0.5ml 0.25M NaOH.

2.2.6. Catalase Assay

Catalase activity was measured by the method of Aebi (Aebi, 1984). The enzyme catalyzes the breakdown of hydrogen peroxide which has an absorption peak at 240nm. By tracing the rate of hydrogen peroxide decomposition, the activity of catalase present in a sample could be calculated.

2.2.6.1. Assay Procedures

Cultured cells were trypsinized and washed twice with PBS. 2 x 10^7 cells were resuspended in 200µl potassium phosphate buffer. The cells were lysed by sonication (Soniprep, MSE, Montana) at 14 microns wavelength for 3 x 20 seconds. The lysate were centrifuged at 12,000g at 4°C for 45 minutes to spin down cell debris. The supernatant was collected and catalase assay was carried out immediately.

100µl sample solution and 2.9ml substrate solution were mixed in a 3ml quartz cuvette. The absorbance at 240nm was monitored by a spectrophotometer (DU 650, Beckman, California) for 1 minute. Potassium phosphate buffer served as the blank.

2.2.6.2. Unit Calculation

One unit of catalase activity is defined as the amount that decomposes 1µmol hydrogen peroxide/min/mg protein at pH 7, 25°C. The molar absorptivity of hydrogen peroxide at 240nm is taken as 39.4 M^{-1} cm^{-1} (Nelson & Kiesow, 1972).

2.2.7. Glutathione Peroxidase Assay

The GPx activities in parental and resistant cells were measured using the method proposed by Paglia and Valentine (Paglia & Valentine, 1967). The glutathione redox system is coupled to the consumption of H_2O_2 and NADPH. GPx catalyzes the conversion of reduced glutathione (GSH) to the oxidized form (GSSG) and *vice versa*. The rate of GSSG formation is an index of GPx activity, which is measured by following the decrease in absorbance of the reaction mixture at 340nm as NADPH is converted to $NADP^+$.

2.2.7.1. Assay Procedures

Cultured cells were trypsinized and washed twice with PBS. 2 x 10^7 cells were resuspended in 200µl potassium phosphate buffer with 1mM EDTA. The cells were lysed by sonication (Soniprep, MSE, Montana) at 14 microns wavelength for 3 x 20 seconds. The lysate were centrifuged at 12,000g at 4°C for 45 minutes to spin down cell debris. The supernatant was collected and GPx assay carried was out immediately.

To a 1ml quartz cuvette 400µl potassium phosphate buffer with 1mM EDTA, 100µl

glutathione reductase solution, 100µl reduced glutathione solution, 100µl NADPH, 100µl sodium azide solution and 100µl sample were added. 100µl substrate solution was added and the absorbance at 340nm was recorded for 2 minutes. Potassium phosphate buffer with 1mM EDTA served as blank. Background oxidation was determined by substituting the sample with equal volume of potassium phosphate buffer with 1mM EDTA.

2.2.7.2. Unit Calculation

One unit of GPx activity is defined as the amount that oxidizes 1nmol NADPH/min/mg protein at pH 7, 25°C. The molar absorptivity of NADPH at 340nm is taken as $6.3 \times 10^3\ M^{-1}\ cm^{-1}$ (Bergmeyer, 1975).

2.2.8. Protein Determination

Protein assays using Bio-Rad Protein Assay Dye Reagent Concentrate (500-0006, Bio-Rad) were based on the method of Bradford (Bradford, 1976). It is a dye-binding assay in which a differential color change of a dye occurs in response to different concentrations of protein.

The microassay procedures for microtiter plates (430247, Corning, Massachusetts) were employed. The linear range of the assay is from 8µg/ml to approximately 80µg/ml. BSA standard solutions were prepared at 0, 10, 20, 40, 80 µg/ml.

160µl of each standard or sample solution was mixed thoroughly with 40µl dye reagent concentrate in separate microtiter plate wells. The mixture was allowed to incubate at room temperature for 15 minutes. Absorbance at 595nm was recorded using a microplate reader (MR 600, Dynatech, Sussex). Protein content in the sample can be determined from the standard curve.

2.3. Statistical Analysis

Due to the small sample size in some groups, Fisher's exact test was used to evaluate the association between two dichotomous variables in the clinical study. To compare the proliferation rate and enzyme activities of cells maintained at different adriamycin concentrations, one-way ANOVA was used to calculate the significance. Normally, $p<0.05$ is regarded as significantly different.

3. RESULTS

3.1. Clinical Study

Biopsy samples were obtained before chemotherapy to confirm diagnosis. Surgical removal of tumour was performed after the patients had received one or more courses of chemotherapy. Both types of samples were subjected to the ABA and P-gp immunohistochemistry. Tumour necrosis was assessed by a pathologist only for samples taken after chemotherapy. All the results are described as followed.

3.1.1. Patients Recruitment

A total of 26 osteosarcoma patients were recruited into our clinical study during the period 7/1996 to 6/1998. Their age ranges from 7 to 67 years old. 12 (46.15%) of them were between 10-19 years old. There were 13 male and 15 female patients. The male to female ratio was 0.87:1. The site involved in most patients (60.71%) was the distal femur.

The results are summed up in Table 3.1. and the they will be further analyzed in detail in the following sections.

Code	Sex	Age	Site	Necrosis	Adriamycin Binding Assay		P-gp Immunohistochemistry		Status	Follow Up (Months)
					Pre-chemo	Post-chemo	Pre-chemo	Post-chemo		
YCE	F	10	Femur	Good	Sensitive		Low		NED	27
LYM	F	12	Femur	Good	Sensitive		Low		NED	24
YCY	M	12	Tibia	Good	Sensitive		Low	Low	AWD	20
BWY	F	12	Femur	Good	Sensitive		High	High	NED	17
LCK	M	17	Femur	Good	Sensitive				NED	12
LW	F	11	Tibia	Good	Resistant	Sensitive	High	High	NED	9
MLK	F	12	Femur	Good	Resistant	Sensitive	Low	CN	NED	8
LSF	F	9	Tibia	Good	Sensitive		Low	CN	NED	4
NKY	F	7	Femur	Good	Sensitive				NED	5
CCH	M	13	Femur	Good	Sensitive				NED	8
WTF	F	10	Femur	Poor	Sensitive		Low	High	NED	29
KP	F	22	Tibia	Poor	Sensitive		Low	High	DOD	15
MCW	M	24	Pelvis	Poor	Sensitive		Low	High	DOD	9
KSW	F	14	Femur	Poor	Resistant		Low		DOD	5
HB	F	26	Femur	Poor	Resistant	Sensitive	Low	High	AWD	12
TI	M	20	Tibia	Poor	Resistant		Low	High	DOD	5
WCW	M	45	Femur	Poor	Resistant			High	AWD	9
LCS	M	51	Femur	Poor	Sensitive	Resistant	Low	Low	NED	7
SHL	M	17	Tibia	Poor	Sensitive	Resistant	Low	High	NED	7
CKL	F	9	Femur	Poor	Resistant	Resistant	Low	Low	NED	4
HS	M	12	Femur	Poor	Sensitive	Resistant	Low	High	NED	6
CMS	F	61	Femur	Poor	Resistant		Low	High	AWD	7
LWF	M	48	Femur	Poor	Resistant				NED	24
CAK	F	67	Femur	Poor	Resistant				AWD	18
CSY	F	47	Pelvis	Poor	Resistant				AWD	9
CL	M	21	Pelvis	Poor	Resistant				DOD	3

CN *Complete Necrosis*
AWD *Alive With Disease*
DOD *Died of Disease*
NED *No Evidence of Disease*

Table 3.1. Summary of results of clinical study arranged according to tumour necrosis status.

3.1.2. Correlation of Adriamycin Sensitivity to Tumour Necrosis

Pre-chemotherapy ABA was carried out in 26 patients. 14 (53.85%) of them were adriamycin sensitive (Table 3.2.). Among them, 8 (57.14%) had good necrosis and 6 (42.86%) had poor necrosis. 12 (46.15%) patients were adriamycin resistant (Table 3.3.). 2 (16.67%) of them had good necrosis and the other 10 (83.33%) had poor necrosis. Statistical analysis using the Fisher's exact test gave a p value of 0.042. It indicated a statistically significant correlation between pre-chemotherapy adriamycin sensitivity and good necrosis.

Post-chemotherapy ABA was carried out in 7 patients. 3 (42.86%) of them were adriamycin sensitive (Table 3.4.). Among them, 2 (66.67%) had good necrosis and 1 (33.33%) had poor necrosis. 4 (57.14%) patients were adriamycin resistant (Table 3.5.). All (100%) of them had poor necrosis. Statistical analysis using the Fisher's exact test gave a p value of 0.143. There is no statistically significant correlation between post-chemotherapy adriamycin sensitivity and good necrosis.

After chemotherapy, 3 patients had their adriamycin sensitivity increased and 3 decreased. The sensitivity of one patient did not change. He remained adriamycin resistant.

Code	ABA Results	Necrosis
YCE	Sensitive	Good
LYM	Sensitive	Good
YCY	Sensitive	Good
BWY	Sensitive	Good
LCK	Sensitive	Good
LSF	Sensitive	Good
NKY	Sensitive	Good
CCH	Sensitive	Good
WTF	Sensitive	Poor
KP	Sensitive	Poor
MCW	Sensitive	Poor
LCS	Sensitive	Poor
SHL	Sensitive	Poor
HS	Sensitive	Poor

Table 3.2. Pre-chemotherapy adriamycin binding assay showed that 14 patients were adriamycin sensitive. 8 (57.14%) of them had good necrosis and 6 (42.86%) of them had poor necrosis.

Code	ABA Results	Necrosis
LW	Resistant	Good
MLK	Resistant	Good
KSW	Resistant	Poor
HB	Resistant	Poor
TI	Resistant	Poor
WCW	Resistant	Poor
CKL	Resistant	Poor
CMS	Resistant	Poor
LWF	Resistant	Poor
CAK	Resistant	Poor
CSY	Resistant	Poor
CL	Resistant	Poor

Table 3.3. Pre-chemotherapy adriamycin binding assay showed that 12 patients were adriamycin resistant. 2 (16.67%) of them had good necrosis and 10 (83.33%) of them had poor necrosis.

Code	ABA Results	Necrosis
LW	Sensitive	Good
MLK	Sensitive	Good
HB	Sensitive	Poor

Table 3.4. Post-chemotherapy adriamycin binding assay showed that 3 patients were adriamycin sensitive. 2 (66.67%) of them had good necrosis and 1 (33.33%) of them had poor necrosis.

Code	ABA Results	Necrosis
LCS	Resistant	Poor
SHL	Resistant	Poor
CKL	Resistant	Poor
HS	Resistant	Poor

Table 3.5. Post-chemotherapy adriamycin binding assay showed that 4 patients were adriamycin resistant. All (100%) of them had poor necrosis.

3.1.3. Correlation of P-glycoprotein Expression to Tumour Necrosis

Pre-chemotherapy P-gp expression was assessed in 18 patients. Low P-gp expression was found in 16 (88.89%) patients (Table 3.6.). Among them, 5 (31.25%) had good necrosis and 11 (68.75%) had poor necrosis. 2 (11.11%) patients had high P-gp expression (Table 3.7.) and both of them had good necrosis. Statistical analysis using the Fisher's exact test gave a p value of 0.137. There was no statistically significant correlation between pre-chemotherapy low P-gp expression and good necrosis.

Post-chemotherapy P-gp expression was assessed in 14 patients. Low P-gp expression was found in 3 (21.43%) patients (Table 3.8.). Among them, 1 (33.33%) had good necrosis and 2 (66.67%) had poor necrosis. 11 (78.57%) patients had high P-gp expression (Table 3.9.). 2 (18.18%) of them had good necrosis and 9 (81.82%) had poor necrosis. Statistical analysis using the Fisher's exact test gave a p value of 0.547. There was no statistically significant correlation between post-chemotherapy low P-gp expression and good necrosis.

13 patients had the pre-chemotherapy and post-chemotherapy P-gp expression evaluated. P-gp expression was found to have increased in 8 (61.54%) patients after chemotherapy. 5 (38.46%) patients had no change and no patients showed a reduction in P-gp expression. Figures 3.1. and 3.2. show the change in P-gp expression in patient TI after chemotherapy.

Code	P-gp Expression	Necrosis
YCE	Low	Good
LYM	Low	Good
YCY	Low	Good
MLK	Low	Good
LSF	Low	Good
WTF	Low	Poor
KP	Low	Poor
MCW	Low	Poor
KSW	Low	Poor
HB	Low	Poor
TI	Low	Poor
LCS	Low	Poor
SHL	Low	Poor
CKL	Low	Poor
HS	Low	Poor
CMS	Low	Poor

Table 3.6. Pre-chemotherapy P-glycoprotein immunohistochemistry showed that 16 patients had low P-glycoprotein expression. 5 (31.25%) of them had good necrosis and 11 (68.75%) of them had poor necrosis.

Code	P-gp Expression	Necrosis
BWY	High	Good
LW	High	Good

Table 3.7. Pre-chemotherapy P-glycoprotein immunohistochemistry showed that 2 patients had high P-glycoprotein expression. All (100%) of them had good necrosis.

Code	P-gp Expression	Necrosis
YCY	Low	Good
LCS	Low	Poor
CKL	Low	Poor

Table 3.8. Post-chemotherapy P-glycoprotein immunohistochemistry showed that 3 patients had low P-glycoprotein expression. 1(33.33%) of them had good necrosis and 2 (66.67%) of them had poor necrosis.

Code	P-gp Expression	Necrosis
BWY	High	Good
LW	High	Good
WTF	High	Poor
KP	High	Poor
MCW	High	Poor
HB	High	Poor
TI	High	Poor
WCW	High	Poor
SHL	High	Poor
HS	High	Poor
CMS	High	Poor

Table 3.9. Post-chemotherapy P-glycoprotein immunohistochemistry showed that 11 patients had high P-glycoprotein expression. 2(18.18%) of them had good necrosis and 9 (81.82%) of them had poor necrosis.

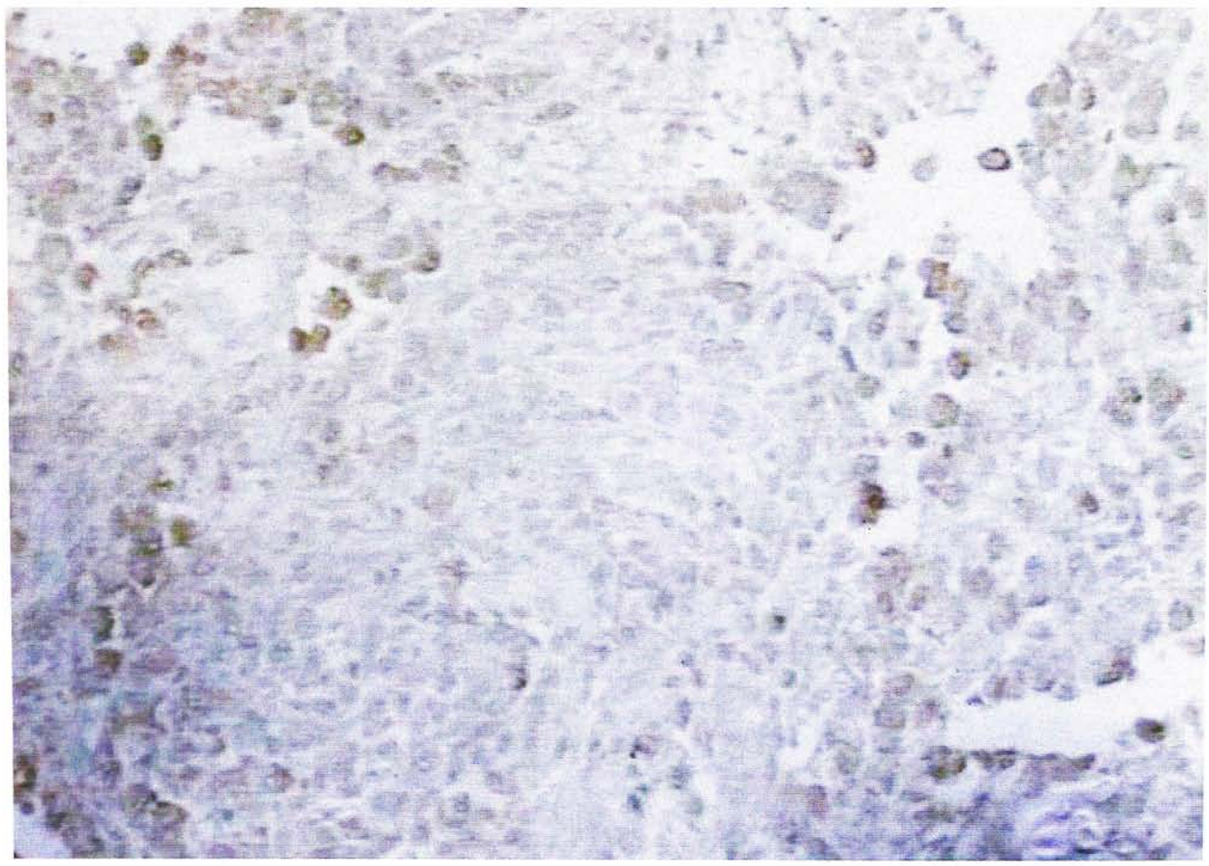


Figure 3.1. A light micrograph showing low P-glycoprotein immunostaining in pre-chemotherapy osteosarcoma from patient TI using diaminobezidine as substrate (200x).

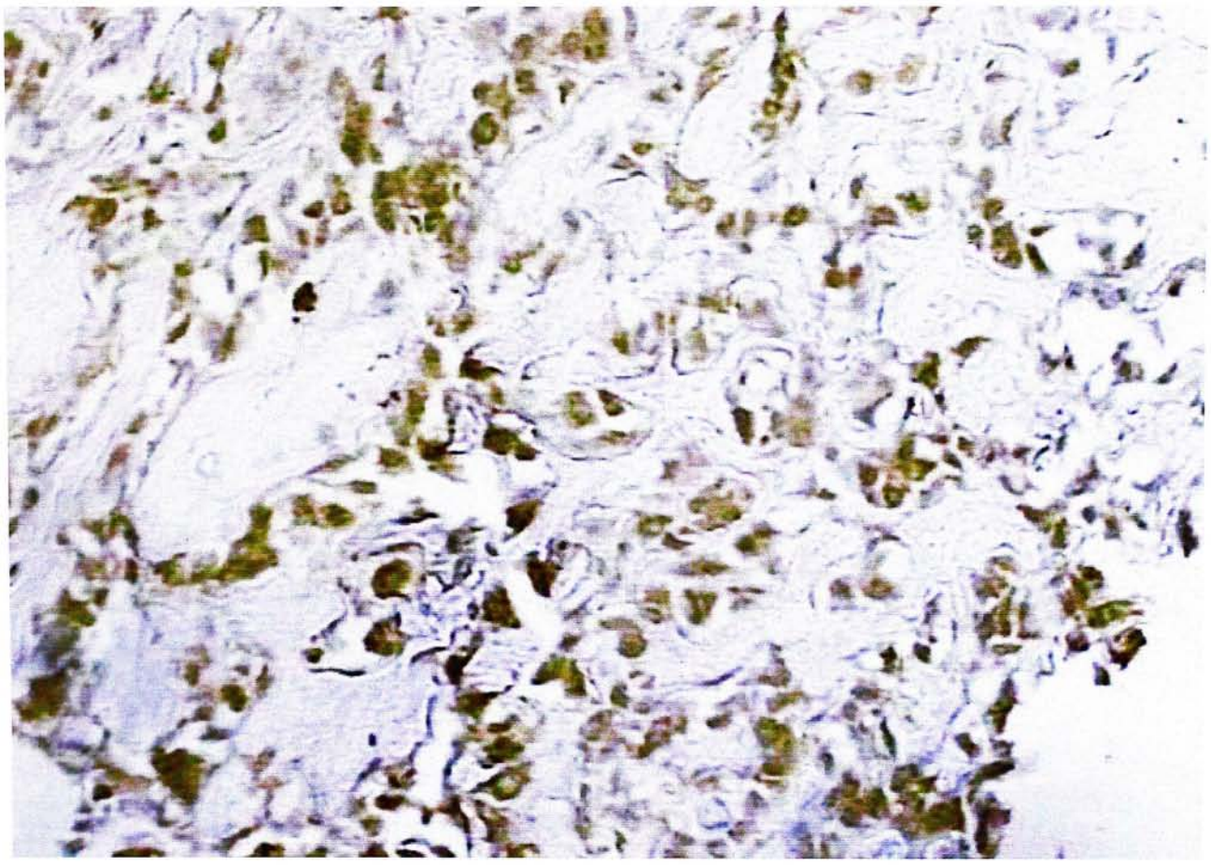


Figure 3.2. A light micrograph showing high P-gp immunostaining in post-chemotherapy osteosarcoma from patient TI using diaminobezidine as substrate (200x).

3.1.4. Correlation of P-glycoprotein Expression to Adriamycin Sensitivity

A total of 23 samples, either pre-chemotherapy or post-chemotherapy, had their adriamycin sensitivity and P-gp expression assessed. 12 samples (52.17%) were adriamycin sensitive (Table 3.10.). Among them, 9 (75%) had low P-gp expression and 3 (25%) had high P-gp expression. 11 (47.83%) samples were adriamycin resistant (Table 3.11.). 8 (72.73%) of them had low P-gp expression and 3 (27.27%) of them had high P-gp expression. Statistical analysis using the Fisher's exact test gave a p value of 0.590. There was no statistically significant correlation between adriamycin sensitivity and low P-gp expression.

Code	ABA Results	P-gp Expression
YCE	Sensitive	Low
LYM	Sensitive	Low
YCY	Sensitive	Low
WTF	Sensitive	Low
KP	Sensitive	Low
MCW	Sensitive	Low
LCS	Sensitive	Low
SHL	Sensitive	Low
HS	Sensitive	Low
BWY	Sensitive	High
LW	Sensitive	High
HB	Sensitive	High

Table 3.10. P-glycoprotein immunohistochemistry was performed on 12 adriamycin sensitive samples. 9 (75%) had low P-glycoprotein expression and 3 (25%) had high P-glycoprotein expression.

Code	ABA Results	P-gp Expression
MLK	Resistant	Low
KSW	Resistant	Low
HB	Resistant	Low
TI	Resistant	Low
LCS	Resistant	Low
CKL	Resistant	Low
CKL	Resistant	Low
CMS	Resistant	Low
LW	Resistant	High
SHL	Resistant	High
HS	Resistant	High

Table 3.11. P-glycoprotein immunohistochemistry was performed on 11 adriamycin resistant samples. 8 (72.73%) had low P-glycoprotein expression and 3 (27.27%) had high P-glycoprotein expression.

3.2. Effect of Adriamycin on Osteosarcoma Cells

ABA and P-gp immunohistochemistry were performed on SaOS-2 cells harvested from the culture to determine the drug sensitivity of SaOS-2 cells as adriamycin concentration in the culture medium was increased. The proliferation rate of the cells was determined by the thymidine incorporation assay. Catalase and GPx activities of cells were measured by the corresponding enzyme assay.

3.2.1. Adriamycin Sensitivity and P-glycoprotein Expression

All SaOS-2 cells cultured, either in the presence of in the absence of adriamycin, were not stained by P-gp immunohistochemistry. However, ABA revealed that the sensitivity of the cells decreased when the drug concentration was stepped up (Table 3.12.).

Adriamycin Concentration in Medium	0ng/ml	20ng/ml	40ng/ml
Adriamycin Sensitivity (%)	18	6	0
P-gp Expression	---	---	---

Table 3.12. Adriamycin sensitivity and P-gp expression were determined in SaOS-2 cells treated with different concentrations of adriamycin. Sensitivity was decreased as adriamycin concentration was increased, although P-gp expression remained undetectable.

3.2.2. Thymidine Incorporation Rate

The change in the amount of thymidine incorporated by SaOS-2 cells after the adriamycin treatment was shown in Figure 3.3. A decrease in the amount of thymidine incorporated was observed when SaOS-2 cells were cultured in the presence of adriamycin. When 20ng/ml adriamycin was added into the culture medium, the amount of thymidine incorporated by the cells dropped to 49.02% of the original. When the drug concentration was stepped up to 40ng/ml, the figure decreased to 20.52% of the original.

Statistical analysis using the 1-way ANOVA gave a p value of less than 0.001. There was statistically significant correlation between the proliferation rate of SaOS-2 cells and adriamycin concentration.

Thymidine Incorporation Rate of SaOS-2 Cell Line Cultured at Various Concentrations of Adriamycin

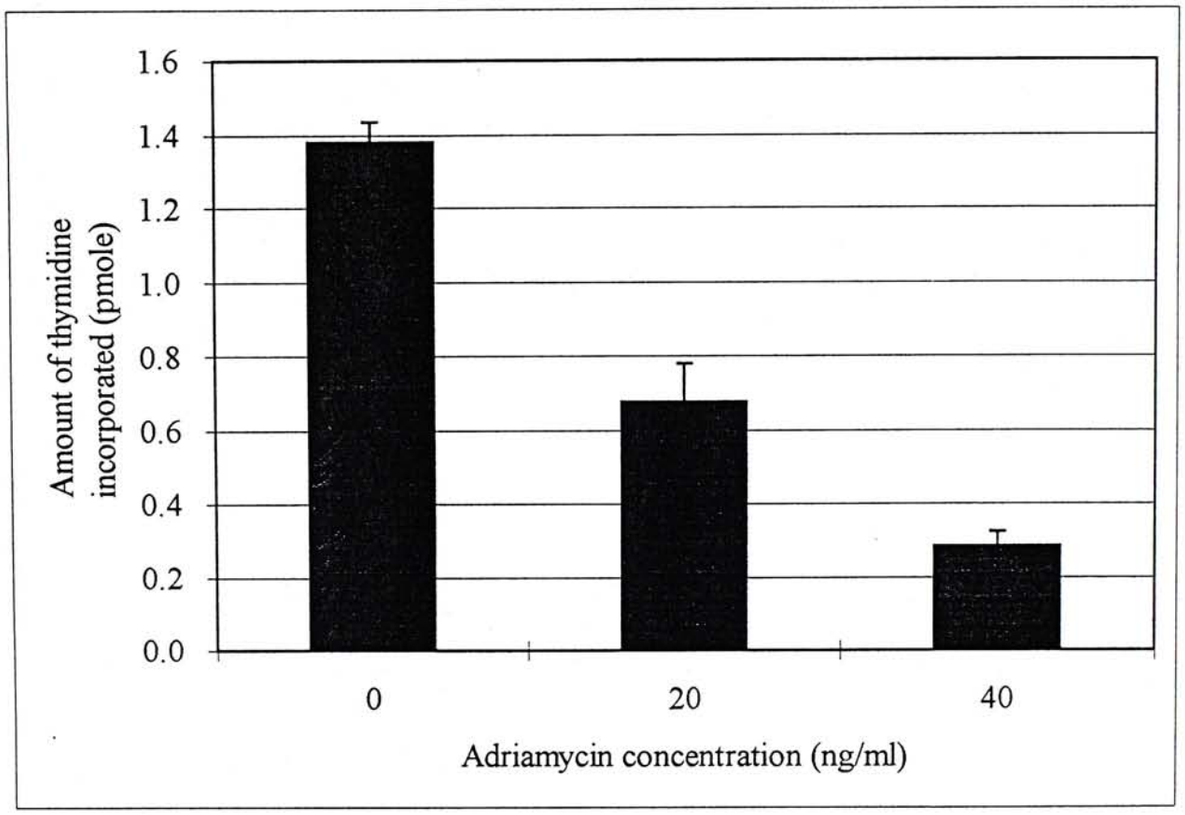


Figure 3.3. A graph showing the amount of thymidine incorporated by SaOS-2 cells cultured at different adriamycin concentrations (n=4). The amount of thymidine incorporated decreased as adriamycin concentration was increased.

3.2.3. Intracellular Concentration of Catalase

The change in catalase activity of SaOS-2 cells after the adriamycin treatment was shown in Figure 3.4. A decrease in the catalase activity was observed when SaOS-2 cells were cultured in the presence of adriamycin. When 20ng/ml adriamycin was added into the culture medium, the catalase activity of the cells dropped to 41.70% of the original. When the drug concentration was stepped up to 40ng/ml, the figure decreased to 22.95% of the original.

Statistical analysis using the 1-way ANOVA gave a p value of 0.127. There was no statistically significant correlation between catalase activity and adriamycin concentration.

Intracellular Catalase Activity of SaOS-2 Cell Line Cultured at Various Concentrations of Adriamycin

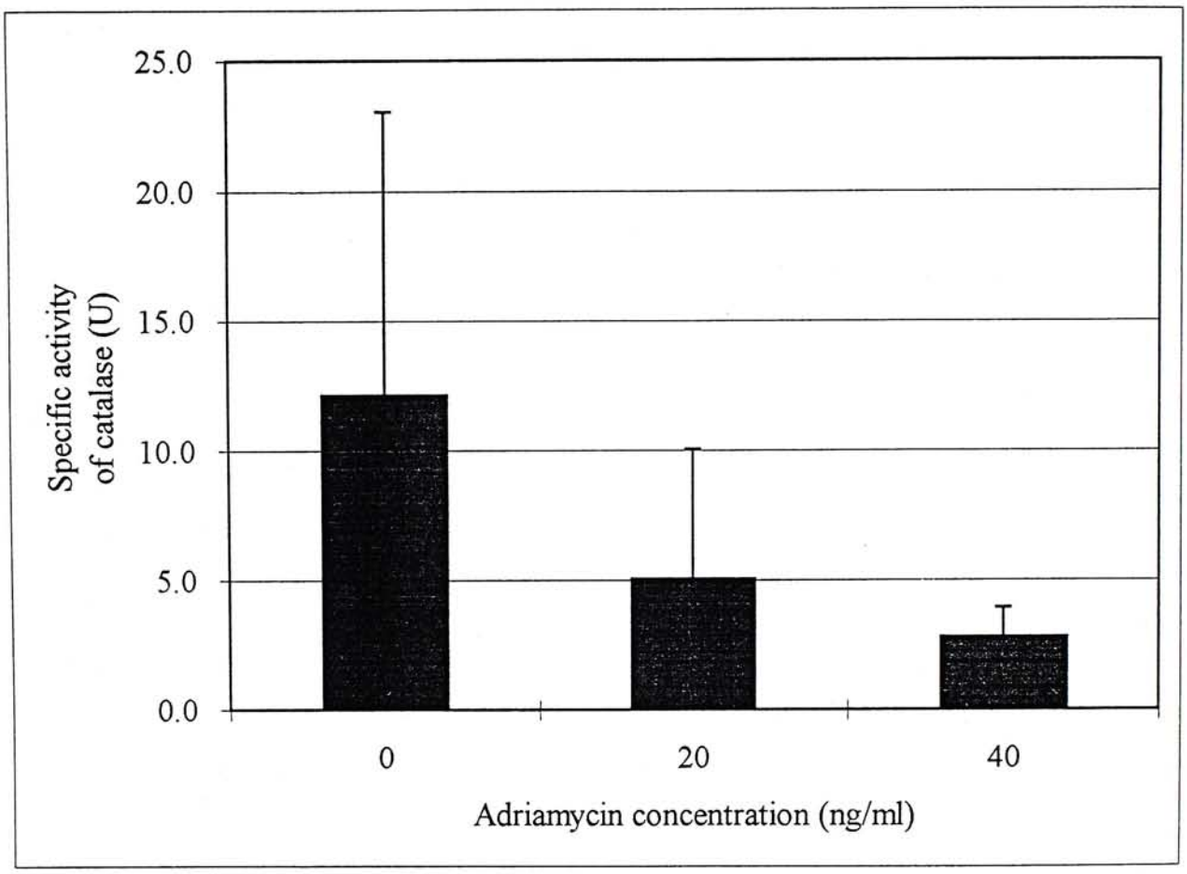


Figure 3.4. A graph showing the catalase activity of SaOS-2 cells cultured at different adriamycin concentrations (n=5). Catalase activity decreased as adriamycin concentrations was increased.

3.2.4. Intracellular Concentration of Glutathione Peroxidase

The change in GPx activity in SaOS-2 cells after the adriamycin treatment was shown in Figure 3.5. A decrease in the GPx activity was observed when SaOS-2 cells were cultured in the presence of adriamycin. When 20ng/ml adriamycin was added into the culture medium, the GPx activity of the cells dropped to 60.46% of the original. When the drug concentration was stepped up to 40ng/ml, the figure decreased to 44.08% of the original.

Statistical analysis using the 1-way ANOVA gave a p value of 0.057. There was no statistically significant correlation between GPx activity and adriamycin concentration.

Intracellular Glutathione Peroxidase Activity of SaOS-2 Cell Line Cultured at Various Concentrations of Adriamycin

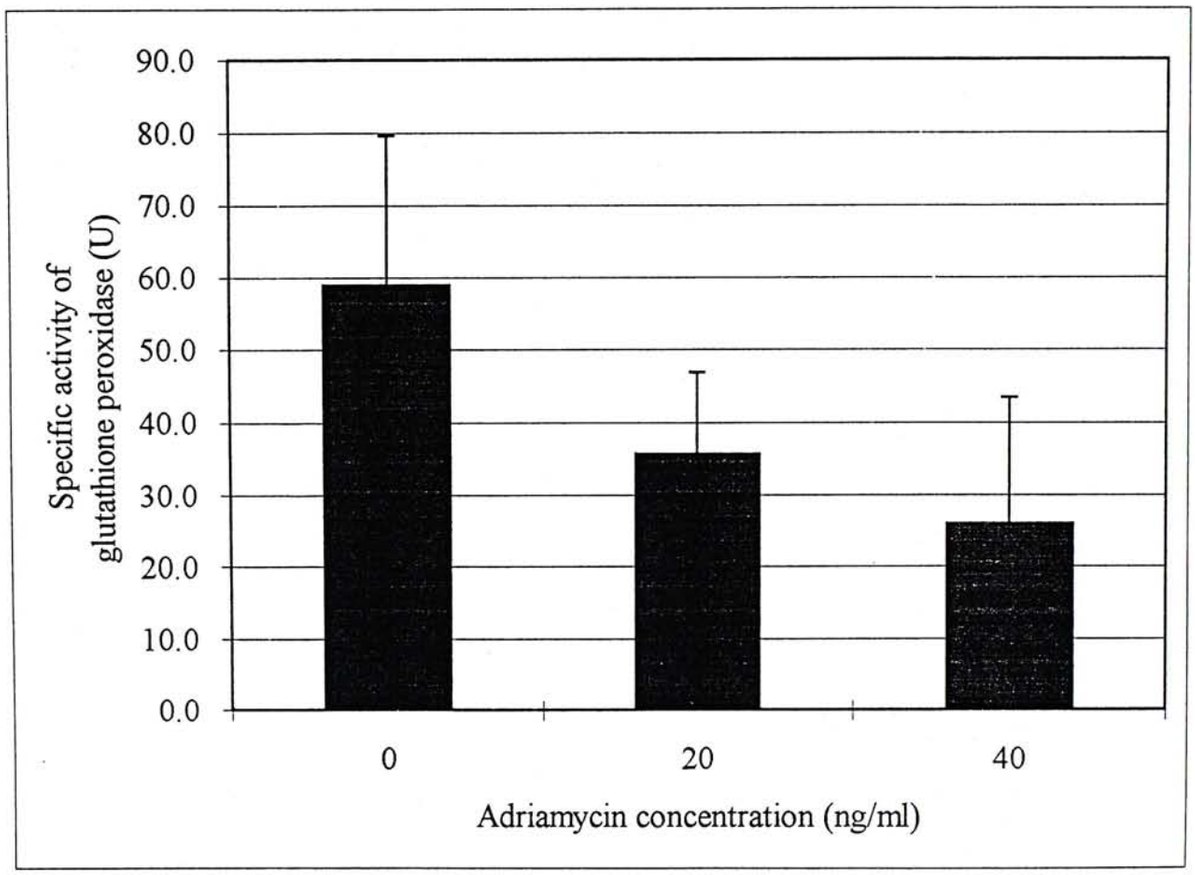


Figure 3.5. A graph showing the glutathione peroxidase activity of SaOS-2 cells cultured at different adriamycin concentrations (n=4). Glutathione peroxidase activity decreased as adriamycin concentration was increased.

4. DISCUSSIONS

4.1. Clinical Study

In order to compare the applicability of the adriamycin binding assay and P-gp immunohistochemistry to predict tumour necrosis in osteosarcoma patients, we have to compare the correlation of ABA results and that of P-gp immunohistochemistry data with tumour necrosis.

4.1.1. Patients Recruitment

Osteosarcoma is the most common primary bone cancer in Hong Kong. In our hospital, 7-10 patients are newly diagnosed and admitted each year. During pre-operative chemotherapy, about 15-20% patients will drop out or succumb. Some patients do not accept surgical treatment and seek alternatives. This makes it difficult to collect post-chemotherapy samples.

Data from the Memorial Sloan-Kettering Cancer Center in the New York City show that the proportion of male to female patients is 1.3:1 (Huvos, 1991). Among our patients, the number of female patients is more than that of male. This may be due to our small sample size. The age distribution and the site involved in most cases matches with what is reported previously by the Memorial Sloan-Kettering Cancer Center.

4.1.2. Correlation between Adriamycin Sensitivity and Tumour Necrosis

The adriamycin binding assay (ABA) is a quick method to determine tumour response towards adriamycin. It is sensitive and easy to perform. The shortcomings are that the specimens must be alive and adequate number of cells must be released from a

tumour in order to verify the accuracy of the assay. Problems may arise when dealing with post-chemotherapy samples as most cancer cells may have already been killed by cytotoxic agents. In this study, the ABA was performed on tumour cells released from fresh osteosarcoma samples. This ruled out the possibility that *in vitro* culture might alter the physiological behaviour of the cells. Besides, quite often, cells may show binding pattern in between the sensitive and the resistant binding patterns. This makes it difficult to classify cells into the sensitive or the resistant phenotype. In such cases, an experienced worker is required to categorize the cells.

Pre-chemotherapy adriamycin sensitivity was found to has statistically significant correlation with tumour necrosis. This means that osteosarcoma patients who are adriamycin sensitive at the clinical onset should have a good tumour necrosis, and *vice versa*. Although ABA is a convenient method to detect clinical drug resistance, very few reports on using it to predict either tumour necrosis or patients survival can be found. Perhaps it is because the use of it on clinical samples has not been studied. It is suggested that pre-chemotherapy ABA should be used as an index to predict the clinical outcome.

Drug resistance may occur either at the onset, as an intrinsic feature of the tumour, or later on, after several courses of chemotherapy. Detection of P-gp activity in pre-chemotherapy tumour samples may not be able to show resistance. Besides, low level of resistance cannot be detected by P-gp immunohistochemistry. Therefore the use of ABA together with an estimation of P-gp expression is recommended to serve as an accurate means of predicting the sensitivity of osteosarcoma to adriamycin (Baldini *et al.*, 1992). This combination also helps to confirm whether the resistance is due to an overexpression of P-gp or not.

The correlation between post-chemotherapy ABA results and tumour necrosis was found to be insignificant. Due to the small sample size, this p value my not be reliable. 3 patients had their adriamycin sensitivity decreased after chemotherapy. We believe that it is because in these patients, drug resistance is induced during chemotherapy, as a result of the cytotoxic pressure exerted by the chemotherapeutic agents. On the other hand, 3 patients had their adriamycin sensitivity increased after chemotherapy. This may be due to the tissue sampling error induced during surgical

operations.

4.1.3. Correlation between P-glycoprotein Expression and Tumour Necrosis in Osteosarcoma

Based on this study, it was found that P-gp in osteosarcoma was induced during chemotherapy, but it cannot be used as a prognostic factor to predict tumour necrosis clinically.

4.1.3.1. P-glycoprotein Is Induced During Chemotherapy

It has been reported that P-glycoprotein (P-gp) overexpression may be an inherent feature of osteosarcoma, soft tissue sarcoma or breast cancer (Serra *et al.*, 1995; Serra *et al.*, 1996; Gregorcyk *et al.*, 1996). Among our patients, P-gp expression was observed before any treatment was given. P-gp was expressed intrinsically in our patients.

Our data showed that before chemotherapy, only 2 out of the 18 (11.11%) samples had high P-gp expression. After chemotherapy, 11 out of 14 (78.57%) samples had high P-gp expression. Among the individual patients, the P-gp expression of 8 patients had increased and that of 5 patients had no change after chemotherapy. No patient had a decrease in P-gp expression. All these data showed that P-gp was induced during chemotherapy.

The induction of P-gp was reported in bladder cancer. Nezasa and his co-workers (Nezasa *et al.*, 1997) reported that in a group of 14 patients who had bladder cancer, clinical specimens were obtained before and after neoadjuvant intra-arterial chemotherapy. The expression levels of *MDR*1 mRNA were significantly higher in the post-treatment specimens than in the pre-treatment specimens. Nakagawa and his co-workers also showed that P-gp mediated multi-drug resistance might be induced after chemotherapy (Nakagawa *et al.*, 1997).

It is thought that in the initial stage of adriamycin exposure, P-gp expression in tumour cells is generally low and overexpression of the protein is not the main factor that confers drug resistance to the tumour (Lee *et al.*, 1996a). As incubation time increased, the tumour cells have to pump out the drug diffused into them in order to survive. P-gp expression is induced and classical MDR results. Increased protein expression can be due to an increased rate of gene transcription or mRNA translation. It is reported that for P-gp, the expression of the protein does not reliably correlate with the level of the *MDR*1 expression (Kandel *et al.*, 1995). Furthermore, in most cases of osteosarcomas, *MDR*1 expression is not induced following chemotherapeutic treatment (Lee *et al.*, 1996a). Therefore it is believed that the increased P-gp expression after chemotherapy is due to an enhanced rate of *MDR*1 mRNA translation.

4.1.3.2. P-glycoprotein Cannot Serve As a Prognostic Factor

In human sarcoma MDR may be the result of an overexpression of P-gp and this overexpression may be an inherent feature (Gerlach *et al.*, 1987; Serra *et al.*, 1996). In most cases, *MDR*1 expression is not induced following chemotherapeutic treatment (Lee *et al.*, 1996a). Analysis of P-gp expression reveals that P-gp is undetectable in the two low-grade lesions, grades III and IV. Besides, the percentage of metastatic osteosarcoma showing an increased level of P-gp expression is significantly higher compared to primary lesions (Serra *et al.*, 1995). Detectable P-gp appears to be an important adverse prognostic factor and consistent absence of the protein is associated with a more favourable prognosis (Chan *et al.*, 1990).

In osteosarcoma, Serra and his co-workers reported that P-gp overexpression is associated with a poor clinical outcome, and an evaluation of P-gp expression in clinical samples at the clinical onset might be useful to identify those patients who would have a poor response to chemotherapy (Serra *et al.*, 1995)

It is found that in patients with high-grade osteosarcoma treated with surgery and chemotherapy, overexpression of P-gp after chemotherapy in tumour cells is associated with a significantly increased risk of adverse events and is independent of

the extent of necrosis after preoperative chemotherapy (Baldini *et al.*, 1995). P-glycoprotein status and the extent of tumour necrosis after preoperative chemotherapy are independent predictors of clinical outcome.

In our study, we found that the correlation between either pre-chemotherapy or post-chemotherapy P-gp expression with tumour necrosis was not statistically significant, although the protein was induced after chemotherapy. It is believed that P-gp cannot serve as an index of clinical outcome. Although adriamycin can act as a potent acute inducer of functional P-gp (Fardel *et al.*, 1997), in the early phases of resistance development and in inherent resistance present at clinical onset, detection of P-gp may not be able to show resistance (Baldini *et al.*, 1992). This tells the reason why pre-chemotherapy ABA can serve as an index of clinical outcome instead of pre-chemotherapy P-gp expression.

Within our group, 11 patients (78.57%) have high P-gp expression after chemotherapy. It is reported that in ovarian adenocarcinoma, P-gp positivity in post-chemotherapy tumours is a better predictor of patients' survival than the occurrence of persistence or recurrence (Khalifa *et al.*, 1997). In this study, we used tumour necrosis as an index of chemotherapeutic response. Since the follow up period of most patients is within one year, the correlation between P-gp expression and patients' survival was not calculated.

4.1.4. Correlation Between Adriamycin Sensitivity and P-glycoprotein Expression

We found that the correlation between adriamycin sensitivity and P-gp expression in our osteosarcoma samples was not statistically significant. We cannot determine the P-gp expression of a given osteosarcoma sample by measuring its adriamycin sensitivity. The adriamycin sensitivity of our samples was not mainly due to P-gp overexpression. The change in sensitivity may be caused by other mechanisms such as elevated antioxidative enzymes activities or a mutation in the topoisomerase II gene.

4.2. Effect of Adriamycin on Osteosarcoma Cells

To further investigate whether clinical adriamycin resistance is due to an enhanced antioxidative enzymes activities, we studied the effect of adriamycin on the catalase and GPx activities of osteosarcoma cells treated with a low concentration (20ng/ml & 40ng/ml) of adriamycin.

4.2.1. Adriamycin Sensitivity and P-glycoprotein Expression

The adriamycin treated osteosarcoma cells are not resistant variants of the parental cell line. Although ABA revealed that their sensitivity decreased, no P-gp expression was observed. In another study, Lee and his co-workers treated lymphoma cells with adriamycin for 7 days. They reported that during resistance development, P-gp was not overexpressed in the initial stage, and these cells did not contribute to a stable cell line until P-gp was overexpressed (Lee *et al.*, 1996b). Therefore it is believed that the adriamycin treated cells are in the initial stage of resistance development and they do not contribute to a resistant cell line.

In another *in vitro* study on SaOS-2 cells conducted by Baldini and his co-workers (Baldini *et al.*, 1992), it was shown that in the highly resistant variants of the SaOS-2 cell line, the majority of cells showed resistance by ABA but only a few cells had increased expression of P-gp. It is believed that the adriamycin resistant phenomenon is due to mechanisms other than P-gp overexpression. It may be the result of an elevated antioxidative enzymes activities or a mutation in the topoisomerase II gene.

4.2.2. Proliferation Rate

There is a significant reduction in the proliferation rate of cells cultured in the presence of adriamycin. The amount of thymidine taken up by SaOS-2 cells dropped to 49.02% of the original when 20ng/ml adriamycin was added into the culture

medium for 14 days and the amount became 20.52% of the original when the drug concentration was increased from 20ng/ml to 40ng/ml. One reason for this is the cytotoxicity of adriamycin. Most anticancer drugs can cause cell-cycle arrest, especially for those quinone-bearing molecules that can intercalate DNA (de Fraine *et al.*, 1990). To escape from adriamycin injury, cells may stall in a dormant stage, so that they are less vulnerable to the drug. The proportion of cell stay in the dormant stage increased as drug concentration was increased. Therefore, the overall proliferation of the cell population decreased.

Another reason for the retarded growth of adriamycin treated osteosarcoma cells may be the result of cell lipid peroxidation induced by the free radicals adriamycin generated (Doroshow, 1996). Upon reduction, anthracyclines can produce oxyradicals (Figure 4.1.) which can initiate peroxidation chain reaction on different biomolecules. Lipid peroxidation in mammalian cells would always give a complex pattern of aldehydes (Figure 4.2.). One of the major aldehydes formed is the 4-hydroxynonenal (HNE) (Zoliner *et al.*, 1991). HNE is a toxic chemical although the sensitivity towards it strongly depended on the cell type.

It is shown that in mammalian cells treated with a high concentration of HNE, acute toxic effects, such as severe damage and death within 1 hour, are observed. At a low concentration, HNE produces lethal effects upon prolonged incubation (Zoliner *et al.*, 1991). Besides, 4-hydroxyalkenals appear to possess the capacity to block cell proliferation at sub-lethal dose (Schauenstein, 1982; Hauptlorenz *et al.*, 1985). Although the chemical reactions and molecular mechanisms involved are largely unknown, the lipophilicity of the aldehyde is an important parameter for HNE-mediated growth inhibition (Kaneko *et al.*, 1988).

In this study, as the adriamycin concentration was increased, the degree of lipid peroxidation in SaOS-2 cells increased. More HNE was produced and the inhibitory effect of this chemical became more prominent. The end result was an inhibited cell growth as revealed by the thymidine incorporation assay.

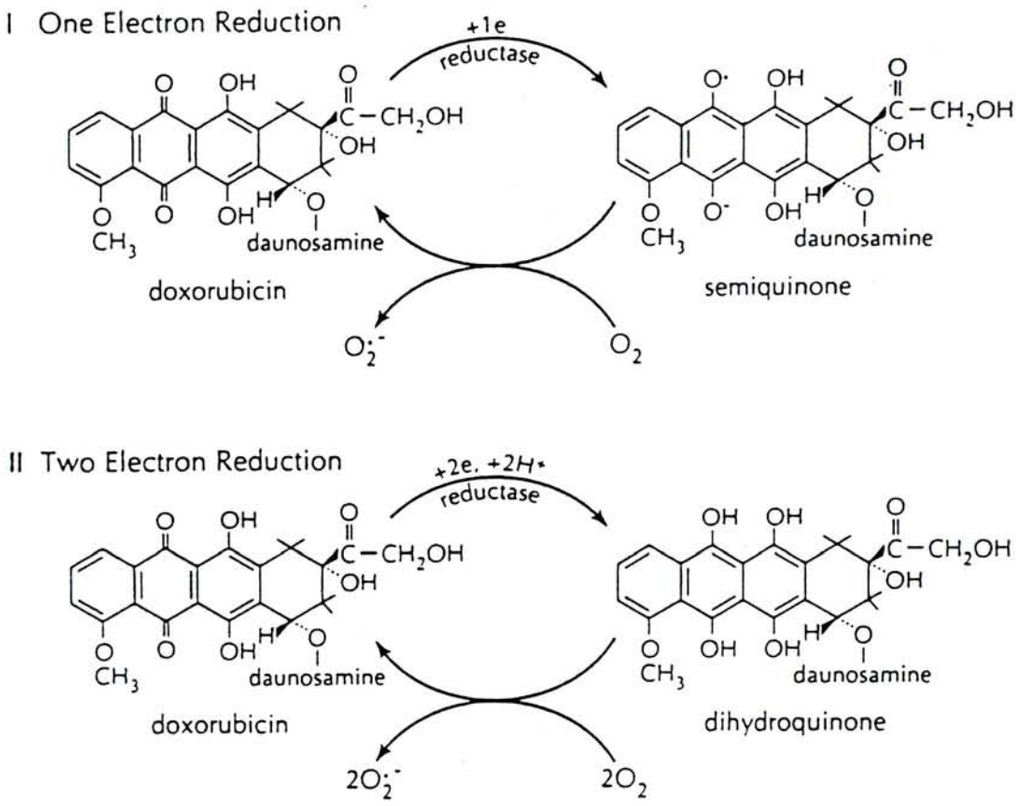


Figure 4.1. One-electron and two-electron reduction of adriamycin (Pratt *et al.*, 1994). One-electron reduction produces semiquinone and two-electron reduction yields dihydroquinone. Both products react rapidly with oxygen to give superoxide anions.

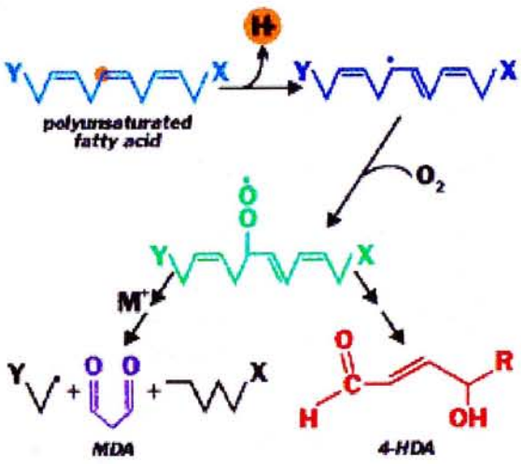


Figure 4.2. Peroxidation of unsaturated lipids yields a broad range of compounds. The two most important products are shown. Malondialdehyde (MDA) is used as an index of the degree of peroxidation and 4-hydroxyalkenals (4-HDA) is the substance that inhibits cell proliferation (R&D, 1996).

4.2.3. Antioxidative Enzymes Activities

The effect of adriamycin on intracellular antioxidative enzymes activities has been studied by many investigators but there is still no consensus. Some authors reported that in a P-gp negative adriamycin-resistant human small cell lung cancer cell line, the levels of superoxide dismutase and GPx were not increased. Catalase activity was only one-third of that measured in sensitive cells (Meijer *et al.*, 1987). Another *in vivo* study showed that chemotherapy treated melanoma did not show any variation in the activities of antioxidant enzymes (Crescimanno *et al.*, 1991a).

In this study, it was found that both catalase and GPx activities were lowered after treatment of adriamycin. Catalase activity dropped to 41.70% of the original when 20ng/ml adriamycin was added into the culture medium and the figure became 22.95% of the original when the drug concentration was increased to 40ng/ml. GPx activity also dropped to 60.46% and then 44.08% of the original. Similar observations were reported by Crescimanno *et al.* in 1991 (Crescimanno *et al.*, 1991b). They found that catalase and total GPx activities were reduced in resistant cells by 18% and 21%. Paranka & Dorr reported a 50% reduction in GPx activity in adriamycin-resistant cultured rat heart cells (Paranka & Dorr, 1994).

Adriamycin mediates the formation of superoxide in microsomal incubations containing NADPH (Handa & Sato, 1975). Since catalase and GPx are inhibited by superoxide (Kono & Fridovich, 1982; Blum & Fridovich, 1985), it is believed that the decrease in the activities of the enzymes is due to the increased production of superoxide by adriamycin. These superoxide ions oxidize the active sites of the antioxidative enzymes, thus reducing their enzyme activity. However, since SOD is responsible for the removal of superoxide ions, an evaluation of SOD activity in the adriamycin treated cells is essential to confirm this hypothesis.

Liebmann and his co-workers showed that a human breast adenocarcinoma cell line MCF-7^{WT} transfected with glutathione peroxidase gene GPX-I were not resistant to adriamycin despite a marked elevation in GPx activity (Liebmann *et al.*, 1995). The protective effect of the antioxidative enzymes may be limited by the over-production of superoxide in this respect. Increased adriamycin tolerance may be effectuated more

efficiently by increased repair of free radical damage than by preventing this damage from occurring (Meijer *et al.*, 1990).

Our data show that GPx and catalase activities are decreased in a similar pattern after adriamycin treatment. If the decrease in thymidine incorporation rate is taken into consideration, it can been seen that the metabolic activities of osteosarcoma cells in the presence of adriamycin may have decreased. This decrease is more or less proportional to the adriamycin concentration. We may assume that the osteosarcoma cells are dying and therefore their anabolism has reduced.

To sum up, it is believed that in this *in vitro* model, both P-gp overexpression and an enhanced rate of free radical removal do not protect osteosarcoma cells from the cytotoxicity of adriamycin.

5. CONCLUSION

Our clinical study shows that pre-chemotherapy adriamycin binding assay can be employed to biopsy samples of osteosarcoma patients before chemotherapy to screen out drug resistant tumours so that alternative treatment can be considered. Adriamycin may induce P-gp overexpression in osteosarcoma which confers MDR to the tumour cells, although not all MDR cases can be explained by P-gp overexpression. Elevation of P-gp expression is due to an enhanced rate of mRNA translation and this increased P-gp expression helps the tumour cells to survive in the presence of cytotoxic drugs. P-gp expression and post-chemotherapy adriamycin sensitivity do not correlate with clinical outcome, and therefore cannot serve as a prognostic factor.

In the cell line model, both the proliferation rate and antioxidative enzymes activities of osteosarcoma cells are lower after the treatment with low concentration of adriamycin. It is believed that free radicals are generated by adriamycin. These free radicals invoke lipid peroxidation and the lipid peroxidation product (4-hydroxyalkenals) in turn inhibits cell growth. On the other hand, excess superoxide ions inhibit the activities of antioxidative enzymes. Moreover, P-gp is not induced during adriamycin treatment, although the cells are resistant as revealed by the ABA. Therefore it is believed that both P-gp and antioxidative enzymes do not protect osteosarcoma cells from the cytotoxicity of adriamycin.

6. FURTHER STUDY

Osteosarcoma is rare compared to other types of cancer. This explains why there are relatively few samples in our study. More reliable conclusions can be drawn only if the sample pool is large. Future studies on osteosarcoma biology rely on the availability of clinical specimens. These samples can be taken from other hospitals by collaboration or by accumulation over a longer period. Besides, a longer follow up period (e.g. over 2 years) is required for a more accurate hypothesis of the association between drug screening methods and clinical outcome of patients.

As mentioned, early detection of MDR not only saves the cost of cancer treatment, but also reduces the side effects brought about by anticancer drugs. Traditional methods used to detect MDR, such as the ABA in our study, utilize the net accumulation of drug in tumour cells as an index of drug resistance. Since biopsy must be performed to take out part of the tumour for analysis, it is invasive and traumatic to the patient. A less invasive and traumatic method to assess drug response in osteosarcoma patients is to be established.

Although there are many proposed mechanisms of action, how adriamycin mediates its cytotoxicity after entering tumour cells remains unknown. It has been shown that free radicals are generated by the drug (Doroshow, 1986), but it is unclear what will happen next. In this study, an evaluation of catalase and GPx has been done, but an association between these enzymes with adriamycin resistance has not been found. However, since catalase and GPx only contributes to a small part in the detoxification of ROS in cells, if the role of free radicals in adriamycin mediated osteosarcoma cytotoxicity is to be established, a more comprehensive assessment of the antioxidative status in adriamycin treated cells is needed.

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8. APPENDIX – SOLUTIONS PREPARATION

Dulbecco's Modified Eagle's Medium (DMEM)

DMEM powder (D-5523, Sigma, St. Louis) was dissolved in 900ml Milli-Q water. The original package was rinsed with a small amount of Milli-Q water to remove all traces of powder. 3.7g $NaHCO_3$ (31437, Merck, Darmstadt) was added to the medium. The pH of the medium was adjusted to 7.2 by 1M HCl and 1M NaOH. Additional Milli-Q water was added to bring the solution to 1L. The solution was sterilized immediately by vacuum filtration using a sterile 0.22μm membrane filter (Millipore, Massachusetts). Sterility of the medium was checked by incubating 5ml of the medium at 37°C overnight. Sterile medium was stored at 4°C.

Iscove's Modified Dulbecco's Medium (IMDM)

IMDM powder (I-7633, Sigma, St. Louis) was dissolved in 900ml Milli-Q water. The original package was rinsed with a small amount of Milli-Q water to remove all traces of powder. 3.7g $NaHCO_3$ (31437, Merck, Darmstadt) was added to the medium. The pH of the medium was adjusted to 7.2 by 1M HCl and 1M NaOH. Additional Milli-Q water was added to bring the solution to 1L. The solution was sterilized immediately by vacuum filtration using a sterile 0.22μm membrane filter (Millipore, Massachusetts). Sterility of the medium was checked by incubating 5ml of the medium at 37°C overnight. Sterile medium was stored at 4°C.

Phosphate Buffered Saline (PBS)

1L 0.15M PBS was prepared by dissolving 8g NaCl (MC), 0.2g KCl (4936, Merck, Darmstadt), 0.2g KH_2PO_4 (4873, Merck, Darmstadt) and 1.15g $Na_2HPO_4 \cdot 2H_2O$ (6580, Merck, Darmstadt) in 950ml double distilled water. After titrating the solution with 1M HCl or 1M NaOH to pH 7.4 at room temperature, the solution was diluted to 1L with double distilled water. The solution was sterilized by autoclaving at 121°C, 15 psi for 30 minutes. Sterile PBS was stored at 4°C.

Fetal Bovine Serum (FBS)

FBS was purchased from Gibco BRL Products (16140-071, Gibco, Maryland). It had been heat inactivated by a thermostatic water bath at 56°C for 30 minutes. 50ml aliquots were dispensed aseptically into sterile bottles and stored at -20°C.

Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture

PSN antibiotic mixture liquid was purchased from Gibco BRL Products (15640-055, Gibco, Maryland). The solution contains 5mg of penicillin, 5mg of streptomycin and 10mg of neomycin in 0.85% saline. 10ml aliquots were dispensed aseptically into sterile bottles and stored at -20°C. 10% PSN was prepared by mixing 1ml PSN with 9ml IMDM. The solution was stored at -20°C.

L-Glutamine Solution

200mM stock L-glutamine solution was prepared by dissolving 0.2922g L-glutamine (G-3126, Sigma, St. Louis) in 10ml DMEM. The solution was immediately filtered through a sterile 0.22μm membrane filter (Millipore, Massachusetts). The sterile L-glutamine solution was stored at -20°C.

Medium for SaOS-2 Culture

The medium was prepared by mixing 440ml DMEM, 50ml FBS, 5ml PSN and 5ml L-glutamine solution aseptically. The resulting medium contains 10% FBS, 1% PSN and 2mM L-glutamine.

Medium for KB-V1 Culture

The medium was prepared by mixing 450ml DMEM and 50ml FBS aseptically. The resulting medium contains 10% FBS in DMEM.

Trypsin-EDTA Solution

Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) was purchased from Gibco BRL Products (25200-056, Gibco, Maryland). The solution contains 2.5g/L of trypsin (1:250) and 0.38g/L of EDTA sodium salt in Hanks' Balanced Salt Solution, without Ca^{2+} and Mg^{2+}. 10ml aliquots were dispensed aseptically into sterile bottles and stored at -20°C.

Trypan Blue Solution

Prepared by dissolving 0.2g trypan blue crystal (T-0776, Sigma, St. Louis) in 50ml PBS. After filtration, the solution was stored at 4°C.

Adriamycin Solution

5ml Milli-Q water was injected into a vial containing 10mg adriamycin (Farmitalia Carlo Erba, Milano) to give a concentration of 2mg/ml. After reconstitution, the solution was drawn out and stored at 4°C.

Vinblastine Solution

10ml sterile normal saline was injected into a vial containing 10mg vinblastine sulphate (Eli Lilly, Indiana) to give a concentration of 1mg/ml. After reconstitution, the solution was drawn out and stored at 4°C.

Collagenase Solution

0.1% collagenase solution was prepared by dissolving 0.01g collagenase II (C-6885, Sigma, St. Louis) in 10ml IMDM. The solution was filtered through a 0.22μm membrane for sterilization. The solution was stored at 4°C.

Fluorescein Diacetate Solution

Fluorescein diacetate solution was prepared by dissolving 0.01g fluorescein diacetate (F-7378, Sigma, St. Louis) in 20ml PBS. The solution was continuously stirred at 60°C under dark condition for 10-15 minutes to facilitate solvation. After filtration through a 0.22μm membrane filter, the solution was stored at -20°C in the dark.

Neutral Buffered Formalin, 10%

Prepared by dissolving 4g $NaH_2PO_4{\cdot}H_2O$ (6346, Merck, Darmstadt) and 6.5g Na_2HPO_4 (6586, Merck, Darmstadt) in 100ml formaldehyde. Distilled water was used to bring the final volume to 1L. The pH of the solution was adjusted to 7.0 using 1M HCl and 1M NaOH.

Formic Acid/Formalin, 10%

100ml formic acid (100264, Merck, Darmstadt) was mixed with 50ml formaldehyde

(4003, Merck, Darmstadt). Distilled water was used to bring the final volume to 1L.

Trypsin Solution

Trypsin solution was prepared by dissolving 0.25mg trypsin (T-8253, Sigma, St. Louis) in 10ml PBS. The solution was stored at 4°C.

Blocking Solution

Blocking solution was prepared by dissolving 0.1g bovine serum albumin (BSA) (A-9647, Sigma, St. Louis) in 10ml PBS. The solution was stored at 4°C.

Primary Antibody

The primary antibody (SC-1517, Santa Cruz, California) is a goat polyclonal antibody. It was raised against a peptide corresponding to amino acids 1251-1269 mapping at the carboxyl terminus of the multiple drug resistance protein 1 (Mdr-1) precursor of human origin. The working concentration of the primary antibody was 1:100. It was stored at 4°C.

Secondary Antibody

The secondary antibody (A-3540, Sigma, St. Louis) was derived from rabbit. It was an anti-goat IgG-HRP conjugate. The antibody was diluted with PBS to 1:100 and stored at 4°C.

Chromogen Substrate Solution

10mg diaminobenzidine hydrochloride (D-5637, Sigma, St. Louis) was dissolved in 10ml PBS. 1ml aliquots were prepared and freezed at -20°C in the absence of light. The chromogen substrate solution is prepared immediately before use. Each aliquot was thawed and 7.5µl of 3% H_2O_2 solution was added as instructed by supplier.

Thymidine Solution

[5'-^{3}H]Thymidine was purchased from Amersham Life Science (TRK328, Amersham, Uppsala) with a specific activity of 14.7Ci/mmol. To prepare the 20µCi/ml working concentration, [5'-^{3}H]thymidine stock solution (1mCi/ml) was diluted by adding 20µl of which into 980µl DMEM. The solution was prepared

freshly each time of assay.

Hepes with BSA Solution

0.25mM Hepes with 2.5mg/ml BSA was prepared by dissolving 14.9mg Hepes powder (H-3375, Sigma, St. Louis) and 0.625g BSA (A-7888, Sigma, St. Louis) in 250ml Milli-Q water. The solution was stored at 4°C.

Scintillation Fluid

One litre of scintillation fluid was prepared by dissolving 4g 2,5-diphenyloxazole (D-4630, Sigma, St. Louis) and 0.2g 1,4-bis[5-phenyl-2-oxazolyl]benzene (P-3754, Sigma, St. Louis) in a mixture of 330ml Triton X-100 and 670ml toluene. The mixture was stirred overnight and stored in a brown bottle at room temperature.

Potassium Phosphate Buffer

0.1M potassium phosphate buffer, pH 7.0, was prepared by diluting a mixture of 6.15ml 0.5M KH_2PO_4 (4873, Merck, Darmstadt) solution and 3.85ml 0.5M K_2HPO_4 (5104, Merck, Darmstadt) solution to 50ml. To prepare potassium phosphate buffer with 1mM EDTA, 18.61mg EDTA disodium dihydrate (ED2SS, Sigma, St. Louis) was dissolved in the buffer.

Glutathione Reductase

Commercial glutathione reductase (359960, Calbiochem, California) at a concentration of 600U/ml was diluted in potassium phosphate buffer with 1mM EDTA to give 2.4U/ml activity. The enzyme solution was stored at 4°C.

Glutathione, Reduced

10mM glutathione (G-6529, Sigma, St. Louis) was prepared by dissolving 15.37mg in 5ml Milli-Q water. The solution was stored at 4°C.

NADPH Solution

Prepared by dissolving 6.25mg NADPH (N-7505, Sigma, St. Louis) in 0.1% $NaHCO_3$ solution (31437, RDH, Seelze). The solution was prepared freshly each time.

Sodium Azide Solution

10mM sodium azide solution was prepared by dissolving 6.5mg NaN_3 (6688, Merck, Darmstadt) in 10ml potassium phosphate buffer with 1mM EDTA. The solution was stored at 4°C.

Substrate Solution for Catalase

30% H_2O_2 solution (18304, RDH, Seelze) was diluted to give 11mM in potassium phosphate buffer. The solution was stored at 4°C in the absence of light.

Substrate Solution for GPx

30% H_2O_2 solution (18304, RDH, Seelze) was diluted to give 1.5mM in potassium phosphate buffer with 1mM EDTA. The solution was stored at 4°C in the absence of light.

ABSTRACT

Osteosarcoma is the most common primary malignant tumour of bone apart from myeloma. Many investigators are searching for prognostic factors to monitor the response of tumours to chemotherapy. Current studies focus on evaluating the reliability of P-glycoprotein expression using immunohistochemistry data. However, its association with response to chemotherapy is controversial since P-glycoprotein immunohistochemistry may only help to detect multidrug resistance in highly resistant tumours. Baldini and his co-workers developed the adriamycin binding assay which is a clinical assay and enables detection of low levels of resistance in tumour cell cultures. In this study, the applicability of P-glycoprotein immunohistochemistry and that of the adriamycin binding assay to predict tumour necrosis were compared.

We found that 46.15% of osteosarcoma samples studied had intrinsic adriamycin resistance as revealed by the adriamycin binding assay. However, P-glycoprotein expression was low in these samples. P-glycoprotein expression was elevated after chemotherapy, which may be the result of increased rate of synthesis. Pre-chemotherapy adriamycin binding assay results correlate at a statistically significant value ($p=0.042$) with tumour necrosis. Post-chemotherapy adriamycin binding assay results and P-glycoprotein expression did not have a statistically significant correlation with tumour necrosis. Moreover, the correlation between adriamycin sensitivity and P-glycoprotein expression in our study was not statistically significant.

In our osteosarcoma samples, P-glycoprotein expression is not the main reason for drug resistance as suggested by the adriamycin binding assay. Alternative mechanisms for drug resistance have been demonstrated. Adriamycin is known to impose growth inhibition on cancer cells. However, its effect on the activities of antioxidative enzymes is controversial. We postulate that intracellular antioxidants might play a role in modulating drug response in osteosarcoma cells. Therefore, we extended our study using an *in vitro* model to investigate the effect of adriamycin on the proliferation rate and the activities of two critical antioxidative enzymes, catalase and glutathione

peroxidase, in an osteosarcoma cell line. In addition, the adriamycin binding assay and P-glycoprotein immunohistochemistry were used to monitor the change of drug sensitivity during drug treatment in this model.

Our *in vitro* study showed that after adriamycin treatment up to 40ng/ml, the osteosarcoma cells developed adriamycin resistance, yet P-glycoprotein expression was not induced. The proliferation rate and the activities of antioxidative enzymes were all suppressed after incubating the cells with sub-concentration of adriamycin. The decrease in proliferation rate and antioxidative enzymes activities was postulated to be due to an inhibition by excess superoxide ions generated by adriamycin through quinone reduction.

In this study, we have clearly demonstrated that pre-chemotherapy adriamycin binding assay is critical for screening out patients who are intrinsically drug resistant. We also find that P-glycoprotein overexpression and enhanced activities of antioxidative enzymes may not be the reasons for adriamycin resistance. Further studies on other mechanisms should be conducted.

撮文

骨肉瘤是除骨髓瘤之外最普遍的原發性惡性骨腫瘤。很多研究員正尋找預後因子用以監察腫瘤在化療期間對藥物的反應。當前研究集中在評估 P-醣蛋白免疫組織化學數據的可信性，但其與藥物反應的關係仍具爭議。而 P-醣蛋白免疫組織化學只能偵測高抗藥性腫瘤對多種藥物的抵抗。Baldini 及其同僚發展了紅霉素結合測試。這項測試已被証明能有效地偵測出培養中癌細胞的低抗藥性。這次研究中，我們比較了 P-醣蛋白免疫組織化學及紅霉素結合測試在預測腫瘤壞死方面的應用能力。

這次研究中紅霉素結合測試顯示百分之 46.15 骨癌樣本原本已具有抗紅霉素能力。這些樣本均有低 P-醣蛋白的表達。P-醣蛋白的表達在化療後可能因其合成加快而上升。化療前紅霉素結合測試結果與腫瘤壞死在統計學上有顯著相關。化療後紅霉素結合測試結果及 P-醣蛋白的表達與腫瘤壞死在統計學上沒有顯著相關 。此外，在我們的骨肉瘤樣本中紅霉素敏感度與 P-醣蛋白的表達在統計學上亦沒有顯著相關。

在我們的骨肉瘤樣本中，P-醣蛋白的表達並非抗藥性的主要原因。先前已有報告指出抗藥性可能由於很多機制，亦已知紅霉素能抑制癌細胞生長，但它對抗氧化酶活性的影響則仍具爭議。我們推測細胞內的抗氧化劑可能幫助調整骨肉瘤細胞的藥物反應，因此我們用一個試管模型來研究紅霉素對骨肉瘤細胞分裂速度及兩種抗氧化酶：催化酶和谷胱甘肽過氧化酶的影響。而紅霉素結合測試及 P-醣蛋白免疫組織化學則用來監察細胞對藥物敏感度的改變。

我們的模型研究顯示經多至 40ng/ml 紅霉素處理後，腫瘤細胞變得抗紅霉素，但 P-醣蛋白的表達並未被誘發。細胞經低濃度紅霉素處理後分裂速度及抗氧化酶的活性均被制止。細胞分裂速度及抗氧化酶活性的下降可能是由於紅霉素醌還原產生過多超氧化離子所致。

是項研究中，我們清楚証明了化療前的紅霉素結合測試能過濾原本已對藥物有抵抗力的病人。我們亦發現過量 P-醣蛋白的表達及抗氧化酶活性的提高可能並非抗紅霉素的原因。未來的研究應考慮其他機制。